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(54) Title: MYCOBACTERIUM TUBERCULOSIS GENES ENCODING PROTEIN ANTIGENS

(57) Abstract

Mycobacterium tuberculosis genes encoding five immunologically relevant proteins have been isolated by systematically screening a lambda gt11 recombinant DNA expression library with a collection of murine monoclonal antibodies directed against protein antigens of this pathogen. One of the *M. tuberculosis* antigens, a 65kD protein, has been shown to have determinants common to *M. tuberculosis* and *M. leprae*. In addition, genes encoding proteins of other mycobacteria (*M. africanum*, *M. smegmatis*, *M. bovis* BCG and *M. avium*) have been isolated. Isolation and characterization of genes encoding major protein antigens of *M. tuberculosis* make it possible to develop reagents useful in the diagnosis, prevention and treatment of tuberculosis. They can be used, for example, in the development of skin tests, serodiagnostic tests and vaccines specific for tuberculosis.

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MYCOBACTERIUM TUBERCULOSIS GENES AND
ENCODING PROTEIN ANTIGENS

Description

Background

05 Tuberculosis was the major cause of infectious mortality in Europe and the United States in the 19th and early 20th centuries. Dubos, R. and J. Dubos, The White Plague: Tuberculosis, Man and Society, Little Brown & Co., Boston, MA, (1952).
10 Today, it remains a significant global health problem.

15 For example, in the United States there are over 20,000 new cases of tuberculosis diagnosed annually. In addition, the steadily declining incidence of tuberculosis evident in preceding years appears to have changed course, reaching a plateau in 1985 and showing an increase in the first half of 1986. Centers for Disease Control, Morbidity/Mortality, Weekly Report, 34:774 (1986); and Centers
20 for Disease Control, Morbidity/Mortality, Weekly Report, 35:774 (1986).

25 Worldwide, tuberculosis remains widespread and constitutes a health problem of major proportions, particularly in developing countries. The World Health Organization estimates that there are ten million new cases of active tuberculosis per year and an annual mortality of approximately three

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million. Joint International Union Against Tuberculosis and World Health Organization Study Group, Tubercle, 63:157-169 (1982).

05 Tuberculosis is caused by Mycobacterium (M.) tuberculosis or Mycobacterium (M.) bovis, which are the 'tubercle bacilli' of the family Mycobacteriaceae. M. bovis is a species which causes tuberculosis in cattle and is transmissible to humans and other animals, in whom it causes tuberculosis. At 10 present, nearly all tuberculosis is caused by respiratory infection with M. tuberculosis. Infection may be asymptomatic in some, but in other individuals, it produces pulmonary lesions which lead to severe debilitation or death. Resistance to 15 tuberculosis is provided by cell-mediated immune mechanisms.

20 Mycobacteria are aerobic, acid-fast, non-spore-forming, non-motile bacilli with high lipid contents and slow generation times. M. leprae is the etiologic agent of leprosy and, among the other mycobacteria, the only major pathogen. Bloom, B.R. and T. Godal, Review of Infectious Diseases, 5:765-780 (1983). However, other mycobacterial species are capable of causing disease. Wallace, R.J. et.al., 25 Review of Infectious Diseases, 5:657-679 (1984). M. avium, for example, causes tuberculosis in fowl and in other birds. Members of the M. Avium-intracellularae complex have become important pathogens among individuals with acquired immunodeficiency syndrome (AIDS). Certain groups of 30

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individuals with AIDS have a markedly increased incidence of tuberculosis-as well. Pitchenik, A.E. et. al., Annals of Internal Medicine, 101:641-645 (1984).

05 Diagnostic and immunoprophylactic measures for mycobacterial diseases have changed little in the past half century. Tuberculin, developed by Koch as a cure for tuberculosis in the late 1800s, is an M. tuberculosis filtrate of complex and poorly-defined composition. It is used as a skin test antigen to detect prior exposure to the bacillus. Enrichment of the protein fraction of this material in the 1930's produced the purified protein derivative (PPD) which is still used to diagnose exposure to 10 tuberculosis. Seibert, F.M. et.al., American Review of Tuberculosis, 30(Suppl.):705-778 (1934). Its usefulness is limited, however, by its lack of specificity and its inability to distinguish active disease from prior sensitization by contact with M. tuberculosis or cross-sensitization to other myco- 15 bacteria. Young, R.A. and R.W. Davis, Proceedings of the National Academy of Sciences, USA, 80:194-20 tuberculosi 1198 (1983).

25 Bacille Calmette Guerin (BCG), an avirulent strain of M. bovis, has been used widely as a live vaccine against tuberculosis for over 50 years. Calmette, A., C. et.al., Bulletin of the Academy of

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Medicine Paris, 91:787-796 (1924). During that time, numerous studies have shown that BCG has protective efficacy against tuberculosis. These studies are reviewed by F. Luelmo in American Review of Respiratory Diseases, 125(pt. 2):70-72 (1982). However, more recently, a major trial of BCG in India indicated that such a vaccine was not protective against tuberculosis in this setting. World Health Organization WHO Technical Report Series, 651 (1980). Presently available approaches to diagnosing, preventing and treating tuberculosis are limited in their effectiveness and must be improved if a solution is to be found for the important public health problem tuberculosis represents worldwide.

Summary of the Invention

The present invention is based on the isolation of genes encoding immunogenic protein antigens of the tubercle bacillus Mycobacterium tuberculosis (M. tuberculosis). Genes encoding such protein antigens have been isolated from a recombinant DNA expression library of M. tuberculosis DNA. Genes encoding proteins of four additional mycobacteria have also been isolated and restriction maps produced.

In particular, genes encoding five immunodominant protein antigens of the tuberculosis bacillus (i.e., those M. tuberculosis proteins of molecular weight 12,000 daltons (12kD), 14kD, 19kD, 65kD and 71kD have been isolated by probing a lambda gt11 expression library of M. tuberculosis DNA with

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monoclonal antibodies directed against M.
tuberculosis-specific antigens.

Recombinant DNA clones producing the specific
antigenic determinants recognized by the monoclonal
05 antigens were also isolated in this manner. DNA
from such recombinant lambda gt11 clones was mapped
with restriction endonucleases; the restriction maps
for genes encoding the five immunodominant protein
antigens (i.e., genes encoding the 12kD, 14kD, 19kD,
10 65kD and 71kD proteins) were deduced. The nucleo-
tide sequence of three of the genes have been deter-
mined and, in each case, the amino acid sequence of
the encoded protein has been deduced.

Brief Description of the Drawings

Figure 1 shows restriction maps of M. tuberculosis DNA. Recombinant DNA clones isolated with
monoclonal antibodies directed against the 12kD,
14kD, 19kD, 65kD and 71kD protein antigens were
mapped with restriction endonucleases. The insert
DNA endpoints are designated left (L) or right (R)
20 in relation to lac Z transcripts which traverse the
insert from right to left. Restriction sites are
represented as follows: A, Sal I; B, BamHI; E,
EcoRI; G, BglIII; K, KpnI; P, PvuI; S, SacI; X, XbaI.
25

Figure 2 shows arrays of antigens from M.
tuberculosis recombinant DNA clones probed with
rabbit hyperimmune serum. The code of the recombi-
nant DNA clones shown on the numbers filter is: 1,
Y3275; 2, Y3274; 3, Y3279; 4, Y3277; 5, Y3247; 6,
30 Y3272; 7, Y3150; 8, Y3254; 9, Y3147; 10, Y3163; 11,

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Y3179; 12, Y3191; 13, Y3252; 14, Y3178; 15, Y3180; 16, Y3143; 17, lambda gt11. Clones 1, 5, 6, 7, 9 and 16 are M. tuberculosis recombinants described in the following section. Clones 10, 11, 14 and 15 are 05 M. leprae recombinants expressing epitopes of the 18kD, 28kD, 36kD and 65kD antigens, respectively. Clones 2, 3, 4, 8, 12, 13 are uncharacterized recombinants from the lambda gt11 M. tuberculosis and M. leprae libraries. Clone 17 is a non-recombinant lambda gt11 control.

Figure 3 shows arrays of recombinant mycobacterial antigens probed with monoclonal antibodies to assess the extent of cross-reactivity between recombinant protein antigen of M. tuberculosis and 15 of M. leprae. The array of clones is identical to that shown in Figure 2. Antibody probes and the antigen sizes recognized are: 1, IT-11 (71kD); 2, IT-31 (65kD); 3, IT-16 (19kD); 4, IT-1 (14kD); 5, IT-3 (12kD).

20 Figure 4 shows restriction maps of DNA encoding four proteins (71kD, 65kD, 19kD and 14kD) of M. tuberculosis and four proteins (71kD, 65kD, 19kD and 14kD) of M. bovis BCG. Restriction sites are represented as follows: A, AatII; B, BamHI; C, 25 BcII; D, DraIII; E=EcoRI; G, BglII; H, Hinfl; K, KpnI; P, PstI; S, SalI; V, PvuI and X, XhoI.

Figure 5 is a comparison of restriction maps of the gene encoding the 65kD protein of 6 mycobacteria (M. leprae, M. tuberculosis, M. africanum, M. bovis 30 BCG, M. smegmatis, M. avium). Restriction sites are

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as follows: B, BamH1; K, KpnI; N, SacI; P, PvuI; S, SalI; X, XhoI.

Figure 6 is the nucleotide sequence of the region containing the M. tuberculosis 19kD gene.
05 The deduced amino acid sequence of the encoded protein is also represented (protein start position, nucleotide 1110; protein stop position, nucleotide 1586).

Figure 7 is the nucleotide sequence of the region containing the M. tuberculosis 71kD gene and the deduced amino acid sequence of the encoded protein.
10

Figure 8 is the nucleotide sequence of the region containing the M. tuberculosis 65kD gene.
15 The deduced amino acid sequences of the two long open reading frames are presented in one letter code over (540) or under (517) the appropriate triplets.

Detailed Description of the Invention

The invention described herein is based on the isolation of genes encoding immunogenic protein antigens of the bacillus M. tuberculosis, which is the major etiologic agent of tuberculosis. In particular, it is based on the isolation, using monoclonal antibodies directed against M. tuberculosis-specific antigens, of genes encoding five immunogenic protein antigens of the tuberculosis bacillus; these five antigens are immunodominant. Immunogenic antigens are those which elicit a response from the immune system.
20
25
30 Immunodominant protein antigens are immunogenic

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antigens against which the immune system directs a significant portion of its response. Genes encoding M. tuberculosis antigens of molecular weight 12,000 daltons (12kD), 14kD, 19kD, 65kD and 71kD were
05 isolated in this manner.

Isolation and characterization of major protein antigens of M. tuberculosis, as described herein, make it possible to develop more effective tools for the prevention, diagnosis, and treatment of tuberculosis.
10 Identification and isolation of genes encoding five immunodominant M. tuberculosis protein antigens, as well as of the five protein antigens, are described below; uses of the genes and encoded products are also described.

15 M. bovis BCG DNA clones were also isolated for the genes encoding the 71kD, 65kD, 19kD and 14kD proteins. In order to compare M. bovis BCG and M. tuberculosis genes encoding proteins of similar molecular weight, restriction endonuclease maps were
20 determined for DNA segments containing each of the genes. Restriction maps for each of these genes is represented in Figure 4.

In addition, DNA clones were isolated for the genes encoding the 65kD protein from M. africanum,
25 M. smegmatis and M. avium. Restriction endonuclease maps were determined for DNA segments containing each of these genes. The restriction maps for these genes, as well as for the genes encoding the 65kD protein of M. tuberculosis, M. bovis BCG and M. leprae, are represented in Figure 5.

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I. Construction of a recombinant expression library of M. tuberculosis DNA

A recombinant DNA expression library of M. tuberculosis DNA was constructed using lambda gt11. 05 The library was constructed with M. tuberculosis genomic DNA fragments in such a way that all protein-coding sequences would be represented and expressed. Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas and R.W. Davis, 10 Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985).

Lambda gt11 is a bacteriophage vector which is capable of driving the expression of foreign insert DNA with E. coli transcription and translation signals. Lambda gt11 expresses the insert DNA as a fusion protein connected to the E. coli Beta-galactosidase polypeptide. This approach ensures that the foreign DNA sequence will be efficiently transcribed and translated in E. coli. This approach is also useful in addressing the problem of the highly unstable nature of most foreign proteins; fusion proteins are often more resistant to proteolytic degradation than is the foreign polypeptide alone. Lambda gt11 and the E. coli strain used 20 (Y1090) have been described previously. Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 80:1194-1198 (1983); Young, R.A. and R.W. Davis, Science, 222:778-782 (1983). The teachings of these publications are incorporated 25 herein by reference. The library constructed in this manner has a titer of 1×10^{10} pfu/ml. and 30

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contains approximately 40% recombinants with an average insert size of 4kB.

II. Screening of the lambda gt11 M. tuberculosis library with antibody probes

05 Murine monoclonal antibodies to protein antigens of M. tuberculosis were used individually to probe the M. tuberculosis recombinant DNA library. This work is described below and with specific reference to the 65kD antigen in the Exemplification. The antibodies used as probes and the sizes 10 of the antigens to which they bind are shown below.

M. tuberculosis

	<u>Antibody</u>	<u>Antigen</u>
	IT-3	12kD
15	IT-20	14kD
	IT-19	19kD
	IT-27	19kD
	IT-17	23kD
	IT-29	23kD
20	IT-15	38kD
	IT-21	38kD
	IT-23	38kD
	IT-13	65kD
	IT-31	65kD
25	IT-33	65kD
	IT-11	71kD

Engers, H.D. et al., Infectious Immunology,
51:718-720 (1986).

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All monoclonal antibodies were used at approximately 1:200 to 1:300 dilution in 50mM Tris-HCl pH8/150 mM NaCl/.05% Tween 20.

Screening of the lambda gt11 recombinant DNA library was performed as described by Young et al. in Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985), the teachings of which are incorporated herein by reference. One modification was made in the method described by Young and co-workers: 1% bovine serum albumin was used in place of 20% fetal calf serum to decrease background.

Briefly, cloned lambda gt11 recombinants were arrayed on lawns of E. coli Y1090. The phage were grown, antigen expression was induced and the antigens were blotted and probed with serum. Detection of signal-producing plaques was performed with a biotinylated secondary antibody system (Vectastain, Vector Laboratories, Burlingame, CA) or with an alkaline phosphatase conjugated secondary antibody system (Protoblot, Promega Biotecl, Madison, WI), both used according to manufacturer's instructions. Signal-producing clones were isolated using antibodies directed against protein antigens of molecular weight 12kD, 14kD, 19kD and 65kD and 71kD. In each case, similar numbers of clones were isolated in screens of approximately 10^5 recombinant plaques. DNA clones encoding the 23kD and 38kD antigens could not be detected with these antibodies, possibly because the native epitope is modified or topographically complex, or because the

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antigen-antibody interaction is too weak to be recognized by current detection methods.

III. Probing of Arrays of lambda gt11 DNA Clones with Antibody Probes

- 05 0.2 ml of a saturated culture of Y1090 was added to 2.5 ml of molten LB soft agar, poured onto 100 mm plates containing 1.5% LB agar and allowed to harden at room temperature for 10 min. 100 ul of phage plate stock containing approximately 10^{11} pfu/ml of the lambda gt11 DNA clones of interest were placed into alternate wells of 96-well tissue culture plates. A multi-pronged transfer device was placed briefly in the wells containing phage and then touched lightly to the surface of the plate onto which the soft agar had been poured. The plates were then incubated at 42°C for approximately 3 hours, at which point clear plaques approximately 5mm in diameter were visible. The plates were then overlayed with nitrocellulose filters saturated with 10mM isopropylthiogalactoside (IPTG) and incubated at 37°C for 3.5 hours. Subsequent processing of filters for detection of antigen was identical to the procedures described for screening of lambda gt11 library with antibody probes.
- 10 15 20 25 Immunoscreening of the lambda gt11 library to isolate clones reactive with monoclonal antibodies specific for the 65kD antigen is described in the Exemplification.

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IV. Recombinant DNA Manipulation

DNA from recombinant lambda gt11 clones was isolated and mapped with restriction endonucleases by standard techniques. Davis, R.W. et al.,
05 Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980).

Figure 1 shows the genomic DNA restriction map deduced for each of the genes encoding the five M. tuberculosis antigens and illustrates how each of
10 the cloned DNAs aligns with that map. All clones isolated with monoclonal antibodies directed against any single antigen align with a single genomic DNA segment. This indicates that all clones were
isolated because they express the protein of interest
15 rather than an unrelated polypeptide containing a similar or identical epitope. In addition, this result suggests that each antigen is the product of a single gene.

The orientation of each DNA insert in the
20 recombinant clones was determined by restriction analysis. Only among the clones for the 65kD antigen were the inserts found in both possible orientations relative to the direction of lac Z transcription in lambda gt11. This suggests that
25 this protein can be expressed in E. coli from signals independent of those provided by lac Z. Similar results have been obtained for recombinant DNA clones encoding the 65kD antigens of M. bovis and M. leprae. Thole, J.E.R. et al., Infectious Immunology, 50:800-806 (1985); Young, R.A. et al.,
30 Nature, 316:450-452 (1985).

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The nucleotide sequences of three regions of the M. tuberculosis DNA were determined: 1) the region containing the M. tuberculosis 19kD gene; 2) the region containing the M. tuberculosis 71kD gene; 05 and 3) the region containing the 65kD gene. The three sequences are represented in Figures 6-8. Sequences were determined using standard techniques, which are described in the Exemplification.

V. Filter hybridization of Insert DNA

10 Arrays of lambda gt11 clones were created as described above and incubated at 42° for 5 hours. The plates were then overlayed with nitrocellulose filters and placed at 4°C for 1 hour. Probe DNA was labelled with ³²P by nick translation. Filter 15 hybridization was performed as described by Davis et al. in Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980), the teachings of which are incorporated herein by reference. Hybridization conditions were as follows: 50% 20 v/v formamide, 5x SSPE (1x SSPE is 0.18M NaCl, 10mM Na_{1.5}H_{1.5}PO₄, 1mM Na₂ EDTA, pH 7.0), 1x Denhardt's solution (0.02% w/v Ficoll, 0.02% w/v polyvinyl-pyrrolidone, 0.02% w/v bovine serum albumin), 0.3% NaDodSO₄ at 42°C for approximately 16 hours, followed by washing in 2x SSPE, 0.2% NaDodSO₄ at 45°C. 25

VI. Recombinant Antigens Recognized by Rabbit Serum

The response of a second animal to an antigen preparation of M. tuberculosis was assessed by

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examining the reactivity of rabbit anti-M. tuberculosis hyperimmune sera with recombinant antigens. Cloned lambda gt11 recombinants were arrayed on lawns of E. coli and probed with the rabbit hyperimmune serum. Anti-M. tuberculosis hyperimmune serum, produced by repeated immunization of rabbits with M. tuberculosis H37Rv culture filtrate, was provided by J. Bennedsen (Statens Serum Institut, Copenhagen, Denmark). These sera were used at 1:100 dilution.

These sera produced positive signals with lambda gt11 clones encoding each of the five M. tuberculosis epitopes which had been isolated with murine monoclonal antibodies (Figure 2). Particularly strong signals were observed with the 65kD and 71kD antigens (Figure 2). These results demonstrate that mice and rabbits can mount an antibody response to the same protein antigens of M. tuberculosis.

Clones for the five M. tuberculosis antigens were detected at similar frequencies in the lambda gt11 recombinant DNA library. Thus, the number and type of antigen-producing clones isolated with polyclonal serum antibodies should reflect the relative titer and diversity of the individual antibodies in this serum.

To determine whether any of the 5 M. tuberculosis antigens are relatively immunodominant in the rabbit humoral immune response to M. tuberculosis, the M. tuberculosis lambda gt11 recombinant DNA library was screened with the rabbit serum. Forty signal-producing clones were isolated, arrayed on

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lawns of E. coli Y1090 and probed with monoclonal antibodies directed against each of the 5 recombinant M. tuberculosis protein antigens. Remarkably, 17 of the 40 clones (43%) reacted strongly with at least one of the four anti-65kD monoclonal antibodies tested. An additional six clones (15%) reacted strongly with the anti-17kD monoclonal antibody (IT-11). This indicates that a large proportion of the anti-M. tuberculosis antibody present in the rabbit serum was directed against the 65kD antigen of M. tuberculosis and suggests that it is a dominant antigen for the rabbit humoral immune response. Seventeen of the clones did not react with any of the monoclonal antibodies tested, suggesting that the rabbit sera may identify M. tuberculosis proteins not recognized by the murine antibodies.

VII. Antigenic Relatedness of M. tuberculosis and M. leprae Proteins

There is evidence that M. tuberculosis and M. leprae share immunologically important antigens. To assess this further, an investigation of the exact nature of the immunological relatedness among recombinant protein antigens of M. tuberculosis and M. leprae was conducted.

For each of five M. tuberculosis and four M. leprae protein antigens, a single recombinant DNA clone containing most or all of the gene of interest was used to express antigen in the following manner. The recombinant phage clones were arrayed on a lawn

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of E. coli Y1090, which was then grown and induced for antigen expression.

Antigen immobilized on nitrocellulose filters was then probed with 26 individual anti-M. tuberculosis and M. leprae monoclonal antibodies. Figure 3 shows the array of DNA clones used and the results obtained with the anti-M. tuberculosis antibodies IT-1, IT-3, IT-11, IT-16, and IT-31, which recognized proteins of 14kD, 12kD, 71kD, 19kD and 65kD respectively. Table 1 details the full results of these cross-screening experiments, showing the reactivity of antigen expressed from individual recombinant DNA clones with each of the individual monoclonal antibodies. Clones were scored as positive only if the signal produced was clearly greater than the background signal produced by the non-recombinant lambda gt11 clone included in each array.

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TABLE 1
Reactivity of Monoclonal Antibodies with
Recombinant Protein Antigens

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Several conclusions can be drawn from the results shown in Table 1. Among the 11 monoclonal antibodies that recognize a 65kD antigen, 7 react with the 65kD protein from both mycobacteria (IT-31, 05 C1.1, IIH9 (identical to IT-33), IIC8, T2.3, Y1-2, SA2.D7C), one antibody reacts only with the M. tuberculosis 65kD protein (IT-13), and two antibodies react only with the M. leprae 65kD protein (IIIE9 and IIIC8). One antibody, ML30A, 10 cross-reacts with an antigen in E. coli and could not specifically identify antigen-producing clones. These results indicate that the 65kD protein antigens of M. tuberculosis and M. leprae are homologues and share a number of epitopes. In 15 addition to these shared epitopes, however, both 65kD antigens have epitopes that are specific for one species relative to the other.

No cross-reactivity was observed between other antigens of these two mycobacterial species. 20 Because monoclonal antibodies recognize a single epitope and because only one or a few antibodies were available for each antigen, it is not clear whether the 65kD proteins are the only homologous protein antigens of M. tuberculosis and M. leprae. 25 Among the antigens for which lambda gt11 clones have been isolated, the 18kD antigen of M. leprae and the 19kD antigen of M. tuberculosis are of similar size. To determine whether these two antigens are related, the homology of the DNA sequences that encode these 30 antigens was examined. At conditions of moderate stringency, no hybridization was observed between

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the insert DNA and Y3147 (an M. tuberculosis 19kD clone) and Y3179 (an M. leprae 18kD clone). This indicates no significant homology between the DNA sequences of the insert DNAs of these two clones.

05 This result suggests that the M. tuberculosis 19kD and the M. leprae 18kD proteins are unlikely to be homologous.

As a result of the work described, recombinant DNA clones encoding five major protein antigens of M. tuberculosis were isolated through the use of an extensive collection of well-characterized murine monoclonal antibodies. These five proteins were also found to be major antigens in the rabbit humoral immune response to M. tuberculosis. One of 15 these antigens, the 65kD protein, is shared with another other mycobacterial pathogen M. leprae.

Several lines of evidence indicate that the 65kD antigen is among the most immunodominant of the protein antigens of M. tuberculosis. Eleven of the 20 25 different M. tuberculosis and M. leprae monoclonal antibodies examined in this study recognized the 65kD recombinant antigen from one or both mycobacteria. In addition, almost half of the recombinant DNA clones isolated with rabbit polyclonal anti-M. tuberculosis sera express the 65kD 25 antigen, reflecting the predominance of antibody to this antigen in these sera.

Considerable evidence indicates that the 65kD antigen plays an important role in the human response to tuberculosis. Antibodies directed against 30 this protein can be detected in the serum of

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patients with tuberculosis. The 65kD antigen is present in purified protein derivatives (PPD's) of M. tuberculosis, M. bovis, and other mycobacteria. Thole, J.E.R. et al., Infection Immunity, 50:800-806 05 (1985). Finally, helper T cell clones reactive with recombinant 65kD antigen have been isolated from patients with tuberculosis, indicating that this antigen is involved in the cell-mediated as well as the humoral immune response to tuberculosis.

Among the major antigens of the leprosy bacillus, the 65kD antigen appears to elicit antibody and T cell responses similar to those observed for the M. tuberculosis antigen. Both serum antibodies and T cells directed against the 65kD M. leprae antigen have been observed in patients with leprosy. Britton, W.J. et al., Journal of Immunology, 135:4171-4177 (1985); Mustafa, A.S. et al., Nature, 319:63-66 (1986). In addition, T cell clones from leprosy patients have been found to respond to recombinant 65kD protein of M. bovis, as well as to PPD's from both M. bovis BCG and M. leprae. Emmrich, F. et al., Journal of Experimental Medicine, 163:1024-1029 (1986); Shankar, P. et al., Journal of Immunology, 136:4255-4263 (1986). It is interesting to note that in vaccine trials in Asia and Africa, BCG provided significant protection against leprosy, ranging from 20% to 80%. Fine, P., Tubercle, 65:137-153 (1984). An intriguing possibility is that the M. bovis BCG 65kD antigen is 10 15 20 25 30 involved in engendering the immune protection

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provided by this vaccine against M. leprae, as well as against M. tuberculosis.

In addition to the 65kD antigen, there is evidence that the 19kD and 71kD antigens of M. tuberculosis may be particularly important in the immune response to this bacillus. Helper T cell clones from tuberculosis patients have been isolated which respond to the recombinant 19kD protein. The 71kD antigen is recognized by the humoral immune system of both mice and rabbits, and antibody to this antigen has been shown to be a prominent component of hyperimmune anti-M. tuberculosis rabbit sera.

15 VIII. Isolation of DNA Clones for Genes Encoding Proteins of Additional Mycobacteria

Using the procedures described above for isolation of genes encoding M. tuberculosis proteins, genes encoding proteins of additional mycobacteria were isolated. DNA clones containing genes encoding the following proteins were isolated:

	<u>Mycobacterium</u>	<u>Protein</u>	<u>Clone</u>
25	<u>M. bovis</u> BCG	71kD	PL1-101
		65kD	PL1-105
		19kD	PL1-501
	<u>M. smegmatis</u>	14kD	PL1-502
	<u>M. avium</u>	65kD	PL1-206
	<u>M. africanum</u>	65kD	PL1-401
		65kD	PL1-301

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For purposes of comparison, genes encoding the following proteins were isolated for M. tuberculosis and M. leprae:

	<u>Mycobacterium</u>	<u>Protein</u>	<u>Clone</u>
05	<u>M. tuberculosis</u>	71kD	Y3272
		65kD	Y3150
		19kD	Y3147
		14kD	Y3248
	<u>M. leprae</u>	65kD	

10 The following strains were used for this purpose:

	<u>Species</u>	<u>Isolate</u>
	<u>M. leprae</u>	Armadillo isolate (WHO)
	<u>M. tuberculosis</u>	Erdmann strain
15	<u>M. africanum</u>	African clinical isolate
	<u>M. bovis</u> BCG	Danish vaccine strain
	<u>M. smegmatis</u>	MC ² -6
	<u>M. avium</u>	AIDS patient isolate

20 DNA from recombinant lambda gt11 clones was isolated, as described above, and mapped with restriction endonucleases, using standard techniques. Davis, R.W. et al., Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980).

25 Figure 4 presents a comparison of the restriction maps for four genes of M. tuberculosis with the restriction maps for four genes of M. bovis BCG which encode proteins of the same molecular weight. As is evident from the figure, in each case, the

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restriction sites on the two genes (e.g., those on the M. tuberculosis gene and those on the M. bovis gene which encodes a protein of the same molecular weight) are essentially identical. This indicates
05 that the sequence of the genes of the two mycobacteria (at least those encoding these four proteins) are very similar and, therefore, the proteins they encode are also very similar.

Figure 5 presents a comparison of the restriction map for the gene encoding the 65kD protein for the six mycobacteria. As is evident, the restriction maps for the genes encoding the 65kD protein of M. tuberculosis, M. africanum, M. bovis BCG, M. smegmatis and M. avium are essentially identical.
10 The fact that there is no detectable difference among these mycobacteria at the level of the restriction map is an indication that, at least at this level, the encoded proteins are the same.
15

As is also evident, the map of the M. leprae 20 65kD gene has several identical restriction sites in common with those of the other mycobacteria; it also has two sites not found in the other genes and lacks three sites present in the others. This indicates that, at the level of the restriction map, there are 25 similarities in the DNA (and the encoded protein). In addition, however, there are differences apparent at this level.

IX. Diagnostic, Therapeutic and Preventive Applications

30 The isolation of genes encoding major protein antigens of M. tuberculosis makes it possible to

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address problems which presently exist in diagnosing
treating and preventing tuberculosis. Isolation of
genes encoding proteins of other mycobacteria, such
as M. bovis BCG, M. africanum, M. smegmatis and M.
05 avium makes it possible to address similar problems
in diseases which they cause.

The nucleotide sequence of three of the five
genes has been determined. The sequence of the
remaining genes can be determined using well-known
10 methods, such as that of Sanger et al. Sanger, F.
et.al., Proceedings of the National Academy of
Sciences, USA, 74:5463-5467 (1977). The amino acid
sequence of each of the immunodominant proteins has
been deduced from the nucleotide sequence of the
15 three genes and can be done for the others.

Identification and characterization of the
genes for major tuberculosis protein antigens and of
the proteins themselves make it possible to develop
improved reagents for diagnosis and immuno-
20 prophylaxis of tuberculosis. Proteins antigens
encoded by an entire gene, or amino acid sequences
(e.g., peptides, protein fragments) which make up
the antigenic determinant of a M. tuberculosis
25 antigen (i.e., M. tuberculosis-specific antigenic
determinants) may be used in serodiagnostic tests
and skin tests. Such antigens would be highly
specific to the tuberculosis bacillus and the tests
in which they are used would also be highly
specific. Highly specific serological tests would
30 be of great value in screening populations for

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individuals producing antibodies to M. tuberculosis-specific antigenic determinants; in monitoring the development of active disease in individuals and in assessing the efficacy of treatment. As a result,
05 early diagnosis of tuberculosis will be feasible, thus making it possible to institute treatment at an early stage of the disease and, in turn, to reduce the likelihood it will be transmitted.

As a result of the work described, it is also
10 possible to determine which segment(s) of the M. tuberculosis antigen is recognized by M. tuberculosis-specific T cells. A mixture of peptides recognized by helper T cells can serve as a specific skin test antigen useful in assessing
15 immunological status (delayed hypersensitivity) of infected individuals and those with whom they come in contact. This specific skin test antigen would be useful in evaluating rapidly the immunological efficacy of anti-tuberculosis vaccines.

20 It is reasonable to expect that the products encoded by M. tuberculosis genes, particularly those shown to be recognized by helper T cells, are themselves immunogenic and thus useful components of vaccines against tuberculosis. These products
25 include proteins and portions of such proteins (e.g., polypeptides and peptides). For example, one approach to vaccine development is the introduction of genes encoding products (e.g., polypeptides) which provide immunological protection into viruses
30 such as vaccinia virus, or bacteria, such as cultivatable mycobacteria, thus producing a vaccine

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capable of engendering long-lasting and very specific immunity. The genes encoding five immunodominant protein antigens of the tuberculosis bacillus, described herein, are useful for that purpose; genes encoding the 65kD, 19kD and 71kD antigens, or a portion thereof, are particularly valuable in vaccine construction.

Because of the similarities in the DNA encoding similarly-sized proteins and, thus, of the encoded proteins themselves, it is possible that, for example, a vaccine effective against two or more of the mycobacteria can be produced.

EXEMPLIFICATION

Isolation and Analysis of Recombinants Expressing the 65kD M. tuberculosis Antigen

The recombinant DNA library of M. tuberculosis genomic DNA fragments in the lambda gt11 vector was constructed as described above. Recombinant phage lambda RY3143 and lambda RY3146 were used. Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985). Subclones of the mycobacterial DNA inserts in these recombinant phage were constructed in pUC19 or M13mp9 vectors using standard recombinant DNA techniques. Messing, J. and J. Viera, Gene, 19:269-276 (1982). Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

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Monoclonal antibodies specific for the 65kD antigen were obtained from the Immunology of Tuberculosis Scientific Working Group under a grant from the WHO/World Bank/UNDP Special Program for 05 Vaccine Development. These antibodies included IT-13 (WTB-78), IT-31 (SA2D5H4), and IT-33 (MLIIH9). Coates, A.R.M. et al., Lancet, 2:167-169 (1981). Gillis, T.P. and T.M. Buchanon, Immunology, 37:172-178 (1982). Anti-B-galactosidase antibodies 10 were purchased from CooperBiomedical. Polyclonal rabbit antisera directed against a sonicate of M. tuberculosis strain H37Rv were elicited as described by Minden and co-workers. Minden, P. et al., Infect. Immun., 46:519-525 (1984). Results are 15 shown in Table 2.

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TABLE 2: PATTERNS OF ANTIBODY REACTIVITIES^a

	<u>Number of Clones</u>	<u>Reactivity with Antibodies</u>		
		<u>IT-13</u>	<u>IT-31</u>	<u>IT-33</u>
05	27	+	+	+
	1	+	+	+
	2	+	-	+
	3	-	+	+
	1	+	-	-
	2	-	+	-
10	2	-	-	+

^a: Recombinant clones expressing antigens reactive with the 65kD antigen specific monoclonal antibodies IT-13, IT-31, and IT-33 were isolated as described above. For the initial screen, a pool of the three antibodies was used; it contained a 1:1000 dilution of each antibody to screen a total of about 8×10^5 recombinant phage from the lambda gt11-M. tuberculosis library. To determine which monoclonal antibody reacted with which of the 38 plaque-purified recombinants, about 100 pfu of each recombinant phage were inoculated in small spots on a lawn of Y1090. The phage were allowed to grow and induced to synthesize the foreign proteins as described previously. The filters were then reacted with a 1:1000 dilution of one of the monoclonal hybridoma antibodies as described above.

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The lambda gt11-M. tuberculosis library was screened with the monoclonal antibodies specific for the 65kD antigen and clones reactive with them were isolated essentially as described by Young et al.

05 Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985). Briefly, for each 150mm LB plate, 0.6ml of a fresh overnight culture of Y1090 was infected with 1-2
10 $\times 10^5$ plaque forming units of the library. After 3.5-4 hours of growth at 42°C, the plaques were overlaid with a dry nitrocellulose filter which had been saturated with 10mM isopropyl-B-D-thiogalactopyranoside (IPTG). The plates were incubated an additional 3.5-4 hours at 37°C and then removed to
15 room temperature and the position of the filters marked. The filters were washed briefly in TBST (50 mM Tris-HCl, pH 8, 150mM NaCl, 0.05% Tween 20) and then incubated in TBST + 20% fetal calf serum.
After 30 minutes at room temperature, the filters
20 were transferred to TBST plus antibody. For the initial screen, the antibody mix contained a 1:1000 dilution of IT-13, IT-31, and IT-33. The filters were incubated with the antibody solution overnight at 4°C with gentle agitation, washed in TBST and reacted with biotinylated goat anti-mouse immuno-globulin, the Vectastain ABC reagent, and developer as described by the manufacturer (Vector Laboratories). After the color had developed the filters were washed with several changes of water
25 and air dried. Phage corresponding to positive signals were twice plaque purified. To determine
30

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which monoclonal antibodies reacted with which of
the recombinant phage, about 100 pfu of a purified
phage stock were inoculated in a small spot on a
lawn of Y1090 bacteria on an LB plate. The phage
05 were allowed to grow and induced to synthesize the
foreign proteins as described above. The filters
were then reacted with a 1:1000 dilution of one of
the monoclonal antibodies. The subsequent steps
were the same as for the initial screen.

10 Western blot assays were carried out as
follows: Cells containing phage or plasmids in
which the expression of the foreign sequences was
under the control of the E. coli lac gene regulatory
sequences were induced to synthesize the foreign
15 proteins by incubating the cells in the presence of
2.5mM IPTG for 2 hours. Crude lysates of cells
expressing lambda gt11 recombinants were made as
described in Huynh et al. Huynh, T.V. et al., In:
DNA Cloning Techniques: A Practical Approach, (D.
20 Glover, ed.) IRL Press, Oxford, Vol. 1, pp. 49-78
(1985). Crude lysates of cells expressing plasmid
encoded proteins were made by harvesting cells from
overnight cultures and resuspending the cells in 10
mM Tris pH7.5/10 mM EDTA containing 100 ug
25 lysozyme/ml. After 10 minutes at room temperature,
SDS was added to a final concentration of 0.5%. A
protease inhibitor (Trasylol, Boehringer Mannheim)
was added to all crude lysates at a final concen-
tration of 0.3%. The crude protein preparations
30 were electrophoresed on 10% polyacrylamide-SDS
Laemmli gels and the separate proteins electrophor-

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etically transferred to nitrocellulose. Laemmli, U.K., Nature, 227:680-685 (1970). Towbin, H. et al., Proceedings of the National Academy of Sciences, USA, 76:4350-4354 (1979). The immobilized proteins were reacted with a 1:1000 dilution of monoclonal antibody IT-13 in TBST overnight at 4°C. The nitrocellulose filters were then washed, reacted with peroxidase-conjugated goat anti-mouse immunoglobulin, and developed as described by Niman and co-workers. Niman, H.L. et al., Proceedings of the National Academy of Sciences, USA, 80:4949-4953 (1983).

The sequences of 5'-end-labeled restriction fragments of the mycobacterial DNA were determined by a modification of the partial chemical degradation technique of Maxam and Gilbert. Brow, M.A.D. et al., Mol. Biol. Evol., 2:1-12 (1985). Maxam, A.M. and W. Gilbert, Proceedings of the National Academy of Sciences, USA, 74:560-564 (1976). For the M13/dideoxy sequencing studies, Sau3AI fragments from the mycobacterial DNA inserts were subcloned into the BamHI site of M13mp9. Phage DNA was isolated from the M13 recombinants and subjected to the dideoxy chain termination sequencing reactions. Biggin, M.D. et al., Proceedings of the National Academy of Sciences, USA, 80:3963-3965 (1983). Sanger, F. et al., Journal of Molecular Biology, 143:161-178 (1980). The products of the sequencing reactions were electrophoresed on 6% acrylamide/7M urea/0.5-2.5 x TBE gradient sequencing gels. The gels were dried

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under vacuum and exposed to Kodak XRP-1 film. The nucleotide sequences were determined independently for both strands of the mycobacterial DNA.

Computer-aided analyses of the nucleic acid sequences and deduced protein sequences were performed using the Databases and programs provided by the Nucleic Acid and Protein Identification Resources of the National Institutes of Health as well as the programs of Chou and Fasman and Hopp and Woods. Chou, P.Y. and G.D. Fasman, Adv. Enzym., 47:45-148 (1978). Hopp, T.P. and K.P. Woods, Proceedings of the National Academy of Sciences, USA, 78:3824-3828 (1981). The nucleotide sequence of the region containing the M. tuberculosis 65kD gene and the deduced amino acid sequence of the two long open reading frames are represented in Figure 8.

B-galactosidase assays were also carried out. Cells were grown in LB broth or LB broth plus 2.5mM IPTG to an OD₆₀₀ of about 0.3. Crude lysates were made and b-galactosidase activity assayed as described by Miller. Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1972).

25 Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific materials and components described herein. Such equivalents are intended to be encompassed in the scope of the following claims.

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CLAIMS

1. Isolated DNA encoding an immunogenic protein antigen of Mycobacterium tuberculosis.
2. DNA of Claim 1 selected from the group consisting of DNA encoding Mycobacterium tuberculosis protein antigens of molecular weight 71kD, 65kD, 19kD, 14kD and 12kD.
3. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 65kD and recognized by a monoclonal antibody selected from the group consisting of: IT-31; C1.1; IIH9; IIC8; T2.3; Y1-2; SA2.D7C and IT-13.
4. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 19kD and recognized by a monoclonal antibody selected from the group consisting of: IT-10; IT-12; IT-16; and IT-19.
5. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 71kD and recognized by the monoclonal antibody IT-11.

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6. Isolated DNA encoding an antigenic determinant of Mycobacterium tuberculosis protein.
7. DNA of Claim 6 which encodes an antigenic determinant selected from the group consisting of antigenic determinants of Mycobacterium tuberculosis proteins of molecular weight 71kD, 65kD, 19kD, 14kD and 12kD.
8. Isolated DNA encoding an amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein, said protein having a molecular weight of approximately 65kD.
9. Isolated Mycobacterium tuberculosis DNA encoding an immunodominant protein antigen having a molecular weight of approximately 65kD, said DNA selected from the group consisting of:
 - a. the DNA insert of clone Y3141;
 - b. the DNA insert of clone Y3143;
 - c. the DNA insert of clone Y3150;
 - d. the DNA insert of clone Y3253; and
 - e. the DNA insert of clone Y3262.
10. A protein antigen encoded by DNA of Claim 9.
11. A protein antigen of Claim 10, wherein the protein antigen is recognized by a monoclonal antibody selected from the group consisting of IT-31; C1.1; IIH9; IIC8; T2.3; Y1-2; SA2.D7C and IT-13.

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12. Isolated DNA having a nucleotide sequence selected from the group consisting of: a) the nucleotide sequence represented in Figure 6, or a portion thereof; b) the nucleotide sequence represented in Figure 7, or a portion thereof; and c) the nucleotide sequence represented in Figure 8, or a portion thereof.
13. A protein or a peptide selected from the group consisting of: a) proteins or peptides encoded by the nucleotide sequence represented in Figure 6, or a portion thereof; b) proteins or peptides encoded by the nucleotide sequence represented in Figure 7, or a portion thereof; and c) proteins or peptides encoded by the nucleotide sequence represented in Figure 8, or a portion thereof.
14. A peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein, said antigenic determinant being unique to Mycobacterium tuberculosis protein.
15. A peptide encoded by isolated Mycobacterium tuberculosis DNA, said peptide recognized by helper T cells.
16. A peptide encoded by the Mycobacterium tuberculosis DNA insert of clone Y3150 or a portion of said DNA insert.

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17. Isolated DNA encoding a protein of Mycobacterium africanum, the protein having a molecular weight of 65kD.
18. Isolated DNA encoding a protein of Mycobacterium avium, the protein having a molecular weight of 65kD.
05
19. A vaccine comprising DNA encoding Mycobacterium tuberculosis protein in a recombinant vaccine vector capable of expressing said DNA.
10. 20. A vaccine of Claim 19 in which the recombinant vaccine vector is vaccinia virus or cultivatable mycobacteria.
21. A vaccine of Claim 20 in which the DNA encodes the 65kD Mycobacterium tuberculosis protein recognized by the monoclonal antibody IT-13, or
15 a portion of said protein.
22. A vaccine comprising DNA encoding an antigenic determinant unique to Mycobacterium tuberculosis cultivatable mycobacteria capable of expressing said DNA.
20
23. A method of detecting antibody against Mycobacterium tuberculosis in a biological fluid, comprising the steps of:
 - a) incubating an immunoadsorbent comprising a solid phase to which is attached
25

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- 05 immunodeterminant Mycobacterium tuberculosis protein with a sample of the biological fluid to be tested, under conditions which allow the anti-Mycobacterium tuberculosis antibody in the sample to bind to the immunoadsorbent;
- 10 b) separating the immunoadsorbent from the sample; and
- 10 c) determining if antibody is bound to the immunoadsorbent, as an indication of anti-Mycobacterium tuberculosis in the sample.
24. A method of Claim 23 in which the Mycobacterium tuberculosis protein attached to the solid phase has a molecular weight of approximately 65kD.
- 15 25. A method of detecting antibody against Mycobacterium tuberculosis in a biological fluid, comprising the steps of:
- 20 a) incubating an immunoadsorbent comprising a solid phase to which is attached a peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein with a sample of the biological fluid to be tested, under conditions which allow antibody against Mycobacterium tuberculosis to bind to the immunoadsorbent;
- 25 b) separating the immunoadsorbent; and
- 25 c) determining if antibody is bound to the immunoadsorbent, as an indication of the

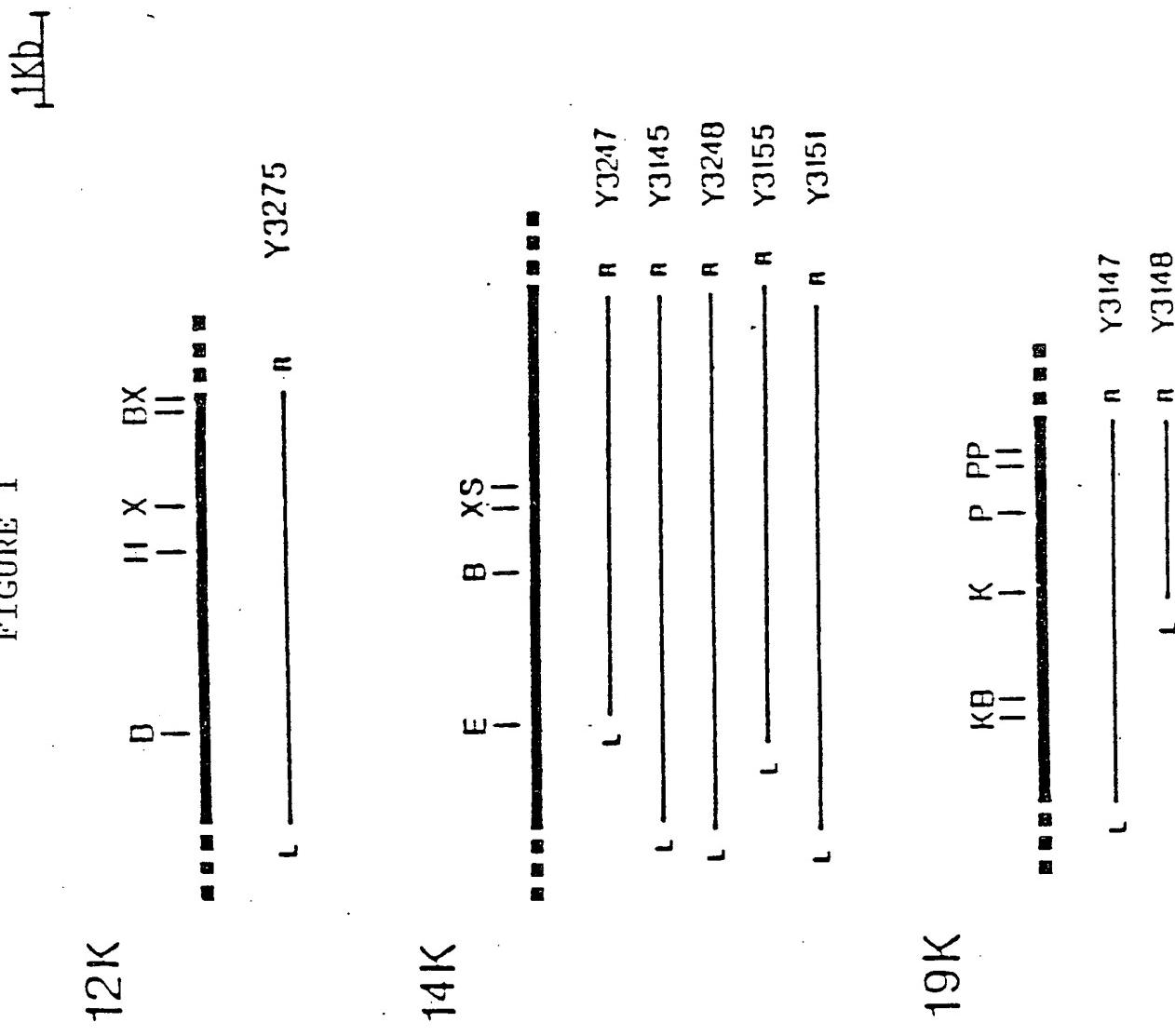
-39-

presence of the antibody against Mycobacterium tuberculosis in the sample.

26. A method of Claim 25 in which the peptide has the amino acid sequence of an antigenic determinant which is unique to Mycobacterium tuberculosis protein.
- 05
27. A kit useful in detecting antibody against Mycobacterium tuberculosis in a biological fluid, comprising a collection of reagents for immunoassay of said antibody, said collection of reagents a solid phase to which is attached immunodeterminant Mycobacterium tuberculosis protein or a peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis.
- 10
- 15

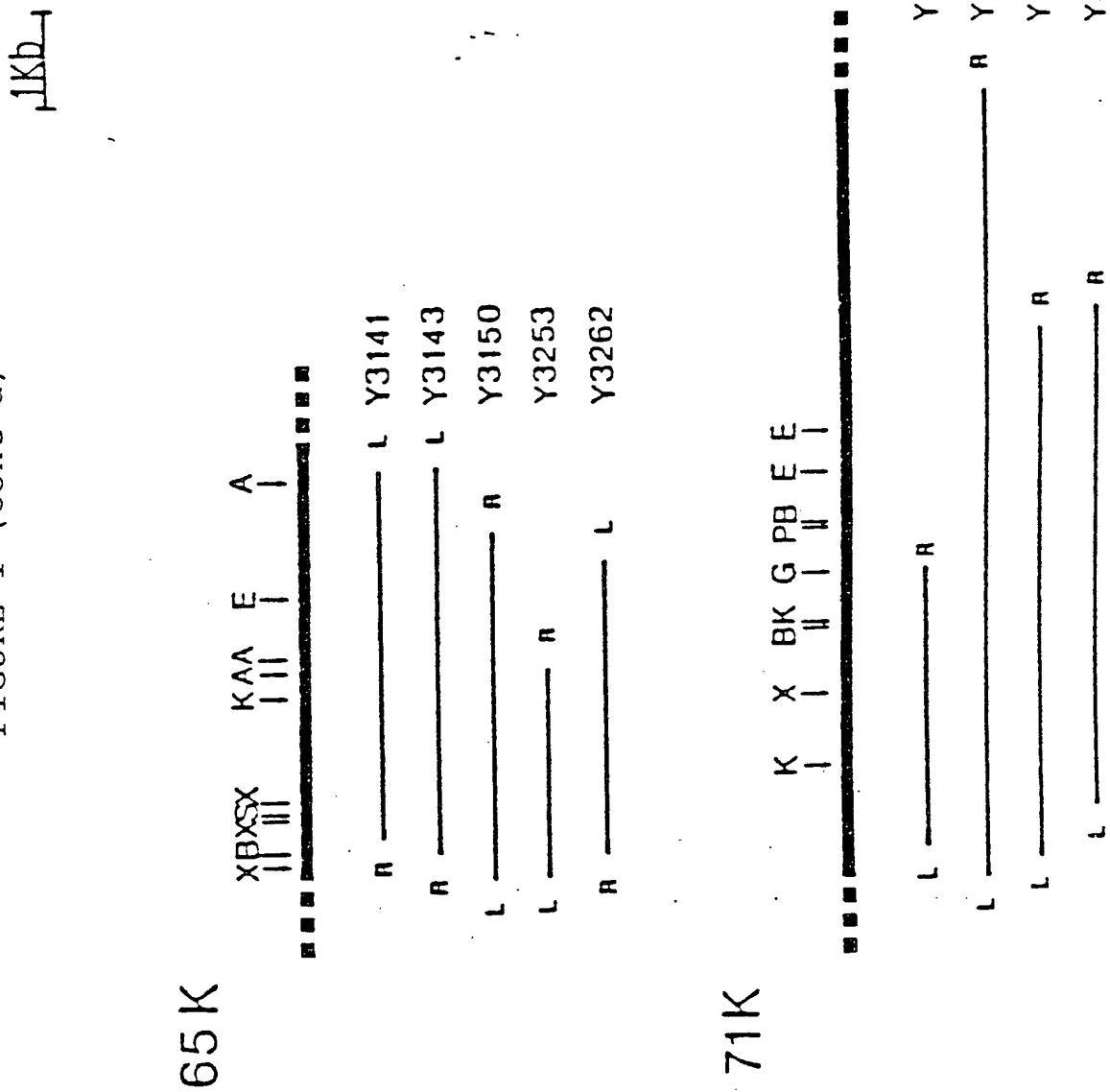
1/43

FIGURE 1



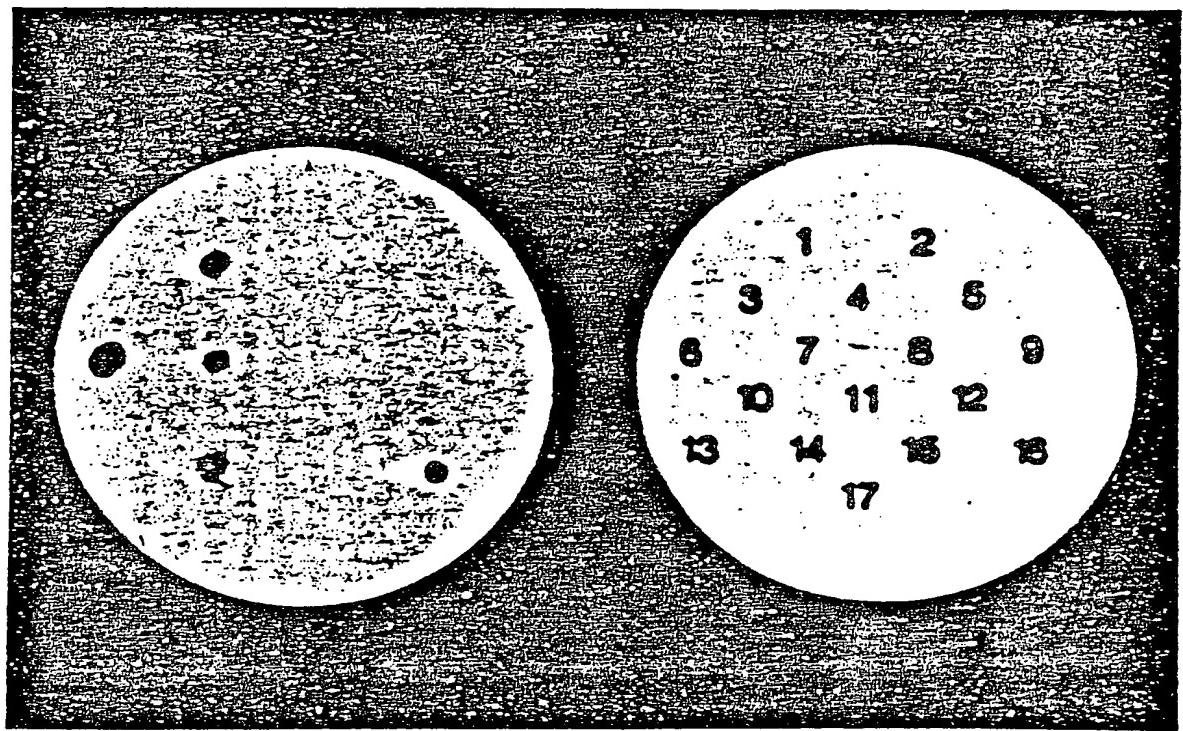
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FIGURE 1 (Cont'd)



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FIG.2



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FIG.3

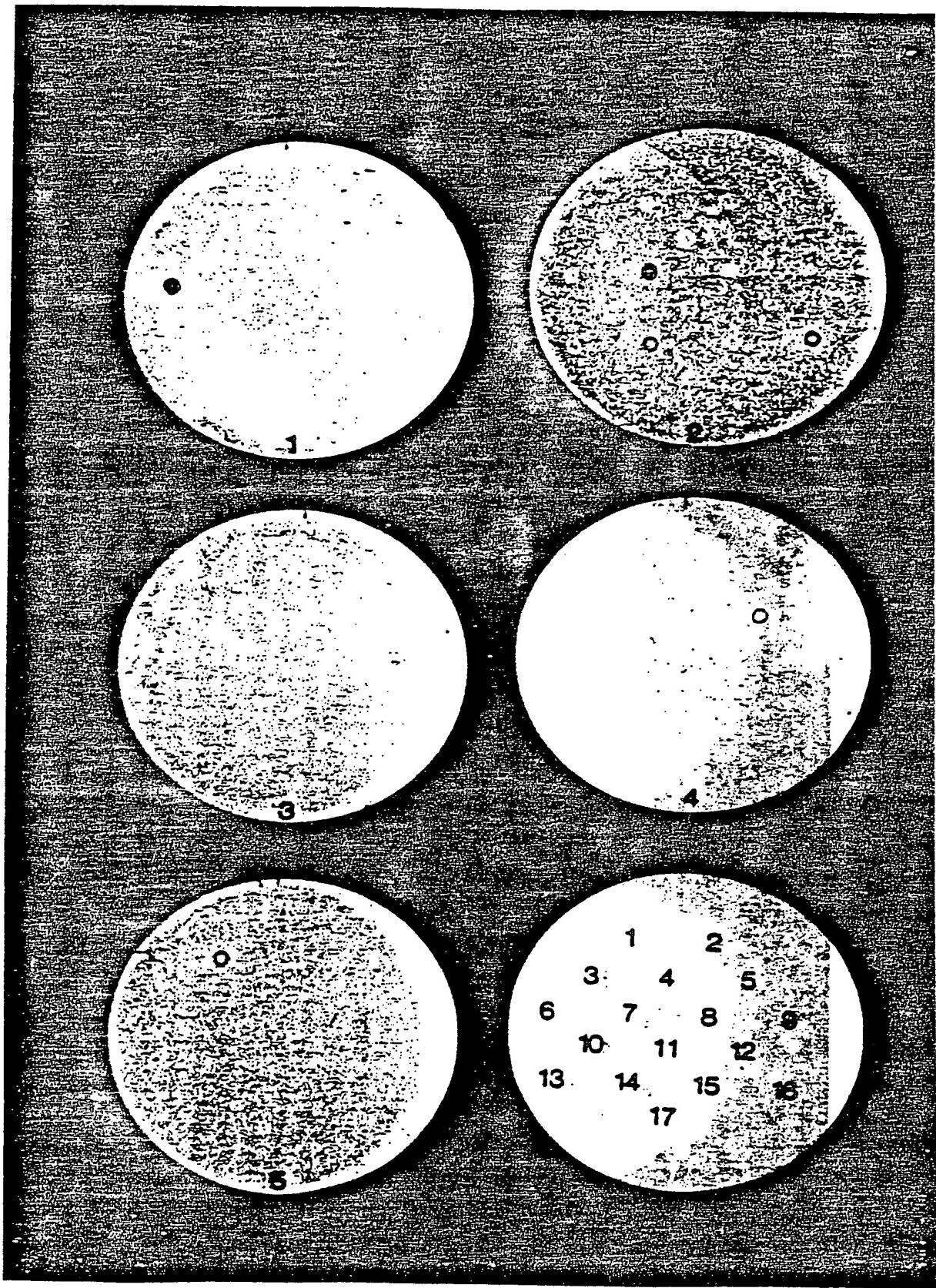
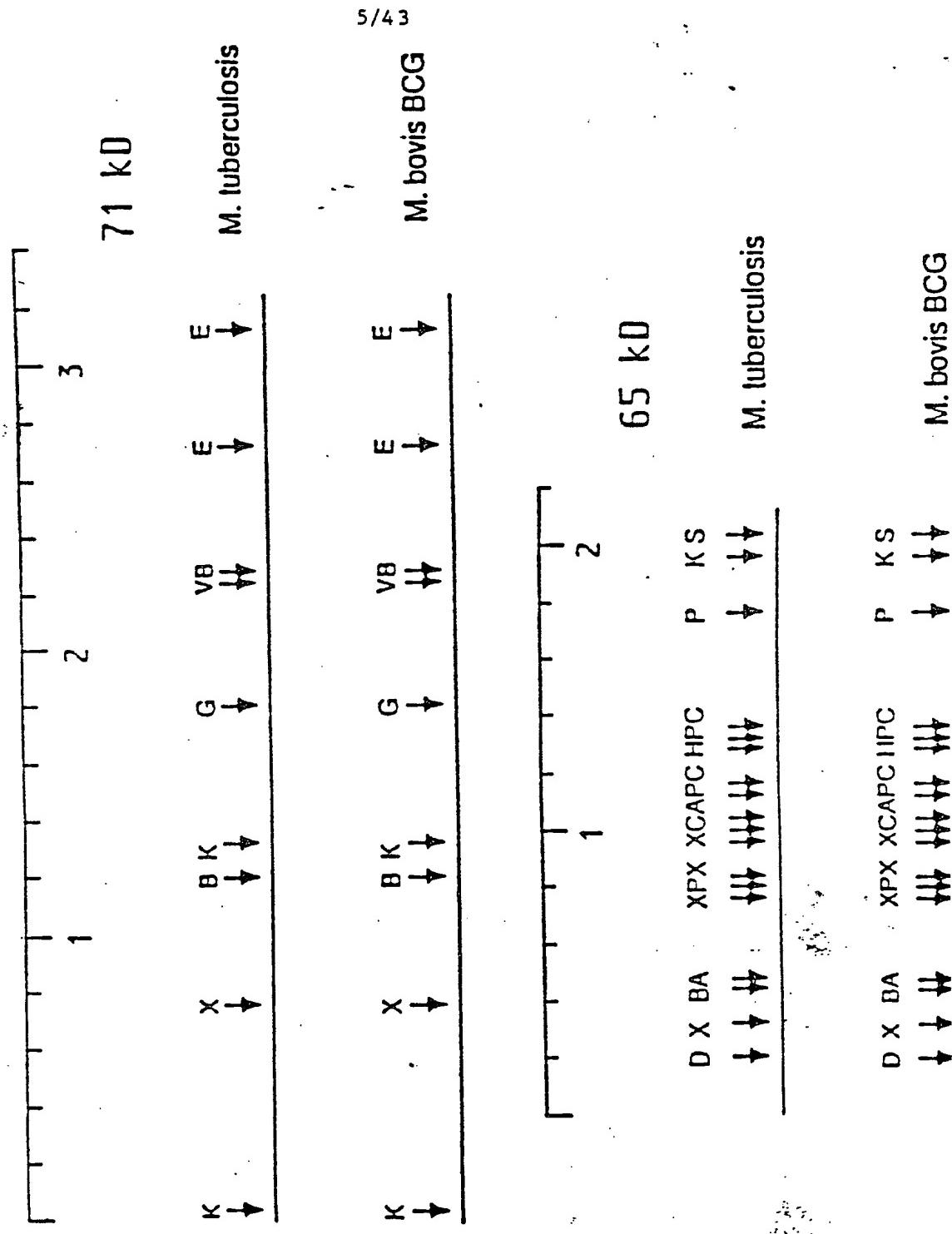
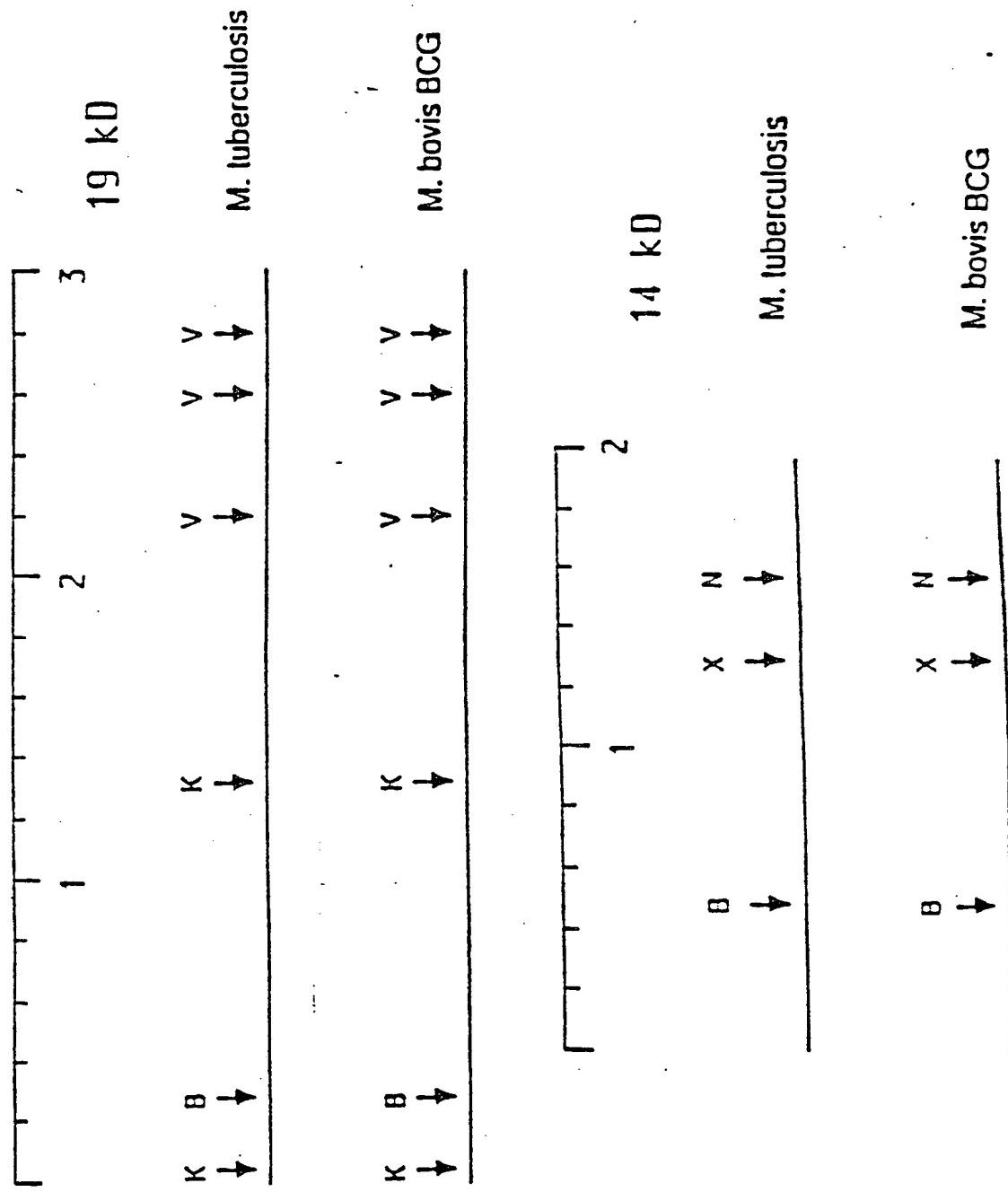


FIGURE 4



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FIGURE 4 (Cont'd)



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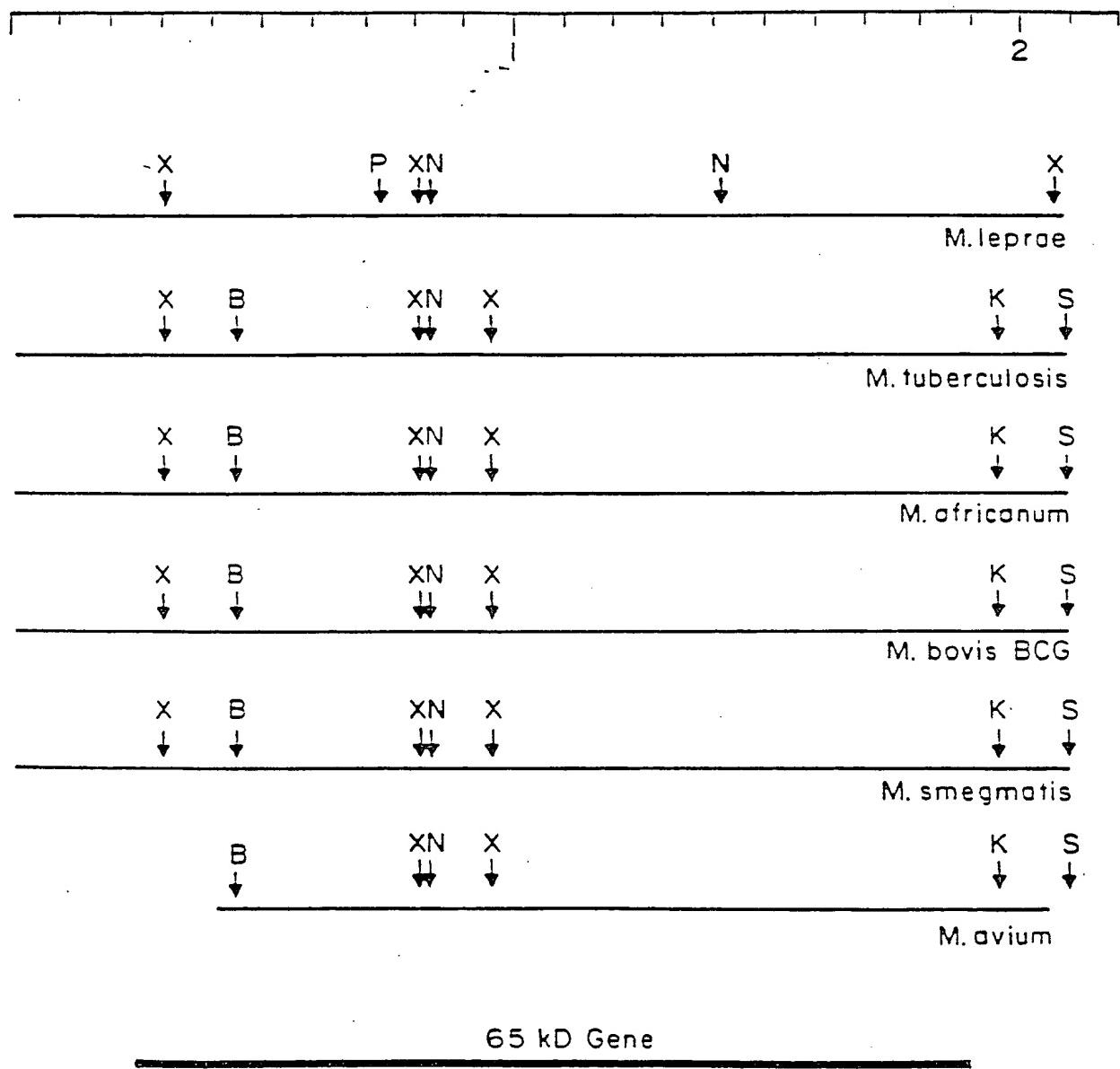


FIG. 5

FIGURE 6

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F	R	V	N	R	L	G	E	I	A	R	P	G	A	R	T	A	H	Q	G
S	V	S	I	A	S	V	R	*	P	D	Q	A	R	G	S	R	T	K	A
P	C	Q	S	P	R	*	D	S	P	T	R	R	A	D	R	A	P	R	R
TTCCGTGTCAATCGCCTCGGTGAGATAGCCCCGACCAGGGATAGGGCATCGGCACCAAGGC																			
10	20	30	40	50	60														
AAGGCACAGTTAGCGGGAGGCCACTCTATCGGGCTTGGTCGGCGCCCTAGCGGGTGGTTCCG																			
E	T	D	I	A	E	T	L	Y	G	S	W	A	R	P	D	R	V	L	A
G	H	*	D	G	R	H	S	L	G	V	L	R	A	S	R	A	G	L	R
R	T	L	R	R	P	S	I	A	R	G	P	A	R	I	A	C	W	P	A
A	A	Q	G	Q	Q	V	V	Q	V	M	R	G	V	F	G	H	A	Q	R
P	R	R	V	S	R	L	C	R	L	C	V	A	F	S	A	M	H	N	A
R	A	G	S	A	G	C	A	G	Y	A	W	R	F	R	P	C	T	T	R
GCCGGCAAGGGTCAGCAGGGTTGTGCAGGGTATGGTGGCTTTCGGCCATGCACAAACGC																			
70	80	90	100	110	120														
CGGGCGGTCCCAGTGTCCAACACGGTCAAATACGGCACCGCAAAGCGGGTACGGTGTGCG																			
G	R	L	T	L	N	H	L	N	H	T	A	N	E	A	M	C	L	A	
R	A	P	D	A	P	Q	A	P	*	A	H	R	K	R	G	H	V	V	R
A	C	P	*	C	T	T	C	T	I	R	P	T	K	P	W	A	C	R	A

FIGURE 6 (CONT'D)

A E A R E I E V H L R R G L G A R G H L
 R K R G K * K C I S A G A S V P G A I W
 G S A G N R S A S P Q G P R C P G P S G
 GCGGAAGCGGGAAATAGAAGTGCATCTCCGAGGGCCATCTG
 130 140 150 160 170 180
 CGCCTCGCCCTTATCTCACGTTAGAGGCCACGGCCCCGGTAGAC
 R F R P F Y F H M E A P A E T G P A M Q
 P L A P F L L A D G C P G R H G P G D P
 S A R S I S T C R R L P R P A R P W R S 9/43
 E L D L H P V D G V G L P G L G D V D R
 N S I S T P S M V W V S P V S V M S T V
 T R S P P R R W C G S P R S R * C R P S
 GAACTCGATCTCCACCCCGTCGATGGTGTGGGTCTCGGTGATGTCGACCGT
 190 200 210 220 230 240
 CTTGAGCTAGAGGTGGGCAGGCTACACACCCAGAGGGCCAGGCCACTACAGCTGGCA
 F E I E V G D I T H T E G T E T I D V T
 V R D G G R R H H P D G R D R H H R G D
 S S R W G T S P T P R G P R S T S R R

FIGURE 6 (CONT'D)

R H D E R N L T G R Q C L P E A A A D V
 G T T S E T S P V D S V C P R P Q P T C
 A R R A K P H R S T V S A R G R S R R A
 CGGCACGGAGCGAAACCTCACCGGTGACAGTGTCTGCCAGGCCGACGCTG
 250 260 270 280 290 300 310 320 330 340 350 360 10/43
 GCCGTTGCTTGCGCTTGGAGTGGCCAGCTGTCAACAGACGGGCTCCGGCTGGCAC
 P V V L S V E G T S L T Q G L G C G V H
 A R R A F G * R D V T D A R P R L R; R A
 C S S R F R V P R C H R G S A A A S T G
 P P E T A R Q H G A V H V A R T A H H R
 P R R P R A N T V P Y M * P A R R I I A
 P G D R A P T R C R T C S P H G A S S P
 CCCCGGAGACGGGCCAACACGGTGGCGTACATGTAGCCCCACGGCCATCATCGC
 310 320 330 340 350 360
 GGGGCCCTGGCGCGGTGTGCCACGGCATGTACATGGGGTAGTAGCG
 G R L G R A L V T G Y M Y G A R R M M A,
 G P S R A G V R H R V H L G C P A D D G
 G S V A R W C P A T C T A R V A C * R R

FIGURE 6 (CONT'D)

R A G V D V F L H G V R G E P L R Q H
 E P A * M F S C T A C A V N P S G A S T
 S R R C F P A R R A R * T P P A P A P
 CGAGCCGGCGTAGATGTTCTGCACGGCGTGCCTGGCAACCCCTCCGGGCCAGCAC
 370 380 390 400 410 420 430
 GCTCGCCGCATCTACAAAGGACGTTGCCGCCACCTTGAGGGAGGGCCGGCTCGTG
 S G A Y I N E Q V A H A T F G E P A L V
 L R R L H K G A R R A R H V G G A G A G
 A P T S T K R C P T R P S G R R W C R
 R H L S R V H V G L G G D A E H P T E M
 A T F P A S T S A W V V T P S T P P K *
 P P F P R P R R P G W * R R A P H R N D
 CGCCACCTTCCCGCGTCCACCGTCCGGCTGGTGGTGAACGCCGAGGCCAACCGAAATG
 430 440 450 460 470 480
 GCGGTGGAAAGGGCGCAGGTGCAGCCGGACCCACTGCGGCTCGTGGGGTGGCTTAC
 A V K G A D V D A Q T T V G L V G G F H
 G G K G R G R R G P H H R R A G W R F S
 W R E R T W T P R P P S A S C G V S I I

FIGURE 6 (CONT'D)

I D M A V G V D D R D H G A V G S A V G
 S T W L W V * M T A T T G R S A P R W A
 R H G C G C R * P R P R G G R L R G G R
 ATCGACATGGCTGTGGGTGTTAGATGACCGCGACCAACGGGGCGTCCGGCTGGGC
 490 500 510 520 530 540 12/43
 TAGCTGTACCGACACCACATCTACTGGCCTGGTGGCTGGCCAGGGGCCACCCG
 D V H S H T Y I V A V V P R D A G R H A
 R C P Q P H L H G R G R P P R S R P P R
 S M A T P T S S R S W P A T P E A T P A
 A I Q V Q R G G H L G G H Q R V D D D
 R Y K S S A A A T S V D T N G S M T I
 D T S P A R R R P P R W T P T G R * R S
 GCGATAACAGTCCAGCGCGGGGCCACCTCGGTGGACACCAACGGGGTCGATGACGAT
 550 560 570 580 590 600
 CGCTATGTTCAAGGTCAAGGTGGCCGGGGAGGCCACCTGTGGCTACTGCTA
 R Y L D L A A A V E T S V L P D I V' I
 S V L G A R R R G G R H V G V P R H R D
 I C T W R P P W R P P C W R T S S S *

FIGURE 6 (CONT'D)

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Q	P	S	V	T	L	N	E	A	D	I	G	D	I	E	S	A	D	L	I	*	
S	P	V	S	P	S	T	K	L	I	L	E	I	S	N	P	R	T	*	*		
A	Q	C	H	P	Q	R	S	*	Y	W	R	Y	R	I	R	G	P	D	R		
CAGCCCAACTGTCACCCCTAACCGAAGCTGATATTGGAGATATCGAATTCCGGGACCTGATA																					
610	620	630	640	650	660																
GTCGGGTACAGTCACGGAGTTGCTTGCCTGACTATAACCTCTATAAGCTTAGGCCCTGGACTAT																					
L	G	T	D	G	E	V	F	S	I	N	S	I	D	F	G	R	V	Q	Y		
A	W	H	*	G	*	R	L	Q	Y	L	Y	R	I	R	P	G	S	L			
G	L	T	V	R	L	S	A	S	I	P	S	I	S	D	A	S	R	I	S		
D	A	R	H	H	L	V	E	A	L	F	R	G	Q	L	G	L	P	P	Q		
M	P	G	T	T	W	*	R	P	C	F	A	V	S	W	D	C	R	H	R		
C	P	A	P	P	G	R	G	P	V	S	R	S	A	G	I	A	A	T	G		
GATGCCGGCACCCACCTGGTAGAGGGCCTGTTTCGGGTCAAGCTGGGATTGCCGCCACAG																					
670	680	690	700	710	720																
CTACGGGGCGTGGACCATTCTCCGGGACAAAGGCCAGTCGACCCCTAACGGGGTGTGTC																					
I	G	P	V	V	Q	Y	L	G	Q	K	A	T	L	Q	S	Q	R	W	L		
H	G	A	G	G	P	L	P	G	T	E	R	D	A	P	I	A	A	V	P		

FIGURE 6 (CONT'D)

A R C W R T S A R N R P * S P N G G C A
 A G M H R C R R G T V E K R V R V V P
 L G C T D V G A A P S R N E Y A S L S H
 W D A P M S A R H R R E T S T R R C P T
 GCTGGATGCACCGATGTCGGCGACCGTCAACGAGTACCGTGTCCCA
 730 740 750 760 770 780
 CGACCCCTACGTTGGCTACAGCCGGCAGCTCTTGCTCATGGCAGCAAACAGGGT
 S P H V S T P A A G D L F S Y A D N D W
 Q S A G I D A R C R R S V L V R R Q G V
 P I C R H R R P V T S F R T R T G C¹⁴₄₃
 H H A T I G S L D H T R G Q R G N E S A
 T T R P S A A L I T H G D S A A M N P R
 P R D H R Q P * S H T G T A R Q * I R D
 CACCACGGGACCATGGCAGGCCATTGATCACACGGGGACAGGGGAATGAAATCCGGG
 790 800 810 820 830 840
 GTGGTGGCTGGTAGCCGGAACTAGTGTGTGCCCGTGTACTTAGGGCC
 V V R G D A A K I V C P S L A A I F G R
 G R S W R C G Q D C V P V A R C H I R S
 W A V M P L R S * V R P C R P L S D A I

FIGURE 6 (CONT'D)

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I G V V E I R C V M Q R * R V F T V C R
 S A S S K S V V S C N G N E C S P C A A
 R R R R N P L C H A T V T S V H R V P P
 ATCGGGCGTCTCGAAATCCGTTGTCACTGCAACGGTAACGAGTGTACCGTGCCGC
 850 860 870 880 890 900
 TAGCCGCAGCAGCTTAGGCAACACAGTACGRTGCCATTGCTCACAAAGTGGCACACGGCG
 D A D D F D T T D H L P L S H E G H A A
 R R R R F G N H * A V T V L T * R T G G
 P T T S I R Q T M C R Y R T N V T H R R
 L D D G S G R F V F H R H Y I A T T T V
 W M T A V G G L C S I G T T L P L L R C
 G * R Q W E V C V P S A L H C H Y Y G A
 CTGGATGACGGCAGTGGAGGGTTGTGTCCATGGCACTACATTGCCACTACCGGTG
 910 920 930 940 950 960
 GACCTACTGCCGTACCCCTCCAAACACAGGTAGCCGTGATGTAACGGTGATGCCAC
 Q I V A T P P K H E M P V V N G S S R H
 P H R C H S T Q T G D A S C Q W * * P A
 S S P L P L N T N W R C * M A V V V T C

FIGURE 6 (CONT'D)

H A G R C R W R T T L P T R K R E F S A
 T P V D A V G E P R Y R P E R E N F P P
 R R * M P L A N H A T D Q K E R I F R R
 CACGCCGGTAGATGCCCGTGGCGAACCCAGCTACCGACCAGAAAGAGAGAATTTCGGCC
 970 980 990 1000 1010 1020
 GTGCCGCCATCTACGGCAACCGCTGGCTGGCGATGGCTGGTCTCTCTAAAGGGGG
 V G T S A T P S G R * R G S L S F K G G
 R R Y I G N A F W A V S W F S L I K R R
 A P L H R Q R V V S G V L F L S N E A A^{16/43}
 .
 A P R P R A L L T R I L P K R S S M P M
 H L D L G P C * R A Y C R S G P Q C R W
 T * T S G P A N A H T A E A V L N A D G
 GCACCTAGACCTGGGCCCTGCTAACGGCATACTGGCGAAGGGTCCCTCAATGCCGATG
 1030 1040 1050 1060 1070 1080
 CGTGGATCTGGAGCCCCGGGACGATTCGGCGTATGACCGGTTACGGCTAC
 C R S R P G Q * R A Y Q R L P G * H R H

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FIGURE 6 (CONT'D)

V * V E P G A L A C V A S A T R L A S P
 G L G R A R S V R M S G F R D E I G I S

D R Y D R Q R S T G * S V D * R S R * P
 T A T T G K G A Q G E A W T D G R G S R
 P L R Q A K E H R \overrightarrow{V} K R G L T V A V A G
 GACCGCTACGGCACAGGAGCAAGGCAAAAGGCAAAAGGCTGAAAGGGTGAACGGTGGACTGACGGTAGCCG
 1090 1100 1110 1120 1130 1140
 CTGGCGATGCTGCCGTTCCTCGTGTCCCACCTCGCACCTGACTGCCAGGCCATCGGC
 V A V V P L P A C P S A H V S P R P L R
 G S R C A F S C L T F R P S V T A T A P
 R * S L C L V P H L T S Q R D R Y G S

E P P F W S Q V F P D V Q A T S R L Q E
 S R H S G R R S F R M F K Q Q V D Y R K
 A A I L V A G L S G C S S N K S T T G S
 GAGCCGCATTCTGGTCCGGCAGGTCTTCCGGATGTTCAAGCMACAAGTCGACTACAGGAA
 1150 1160 1170 1180 1190 1200
 CTCGGCGTAANGACCAGCGTCCAGAAAGGCCTACAAAGTTCTGTTCTAGCTGATGTCCTT
 L R W E P R L D K R I N L C C T S * L F
 A A M R T A P R E P H E L L D V V P L
 R M O N R T K G S T * A V L R S C S A

FIGURE 6 (CONT'D)

A V R P R P R Q A R R Q A P A P P P G R
 R * D H D R G R H D G K P R R L R A E
 G E T T T A A G T T A S P G A A S G P K
 GCGGTGAGGACACGACCACGGCAGGCACGGCAAGGCCGGCCGCTCCGGCCGA
 1210 1220 1230 1240 1250 1260
 CGCCACTCTGGTGGCGCCGGTCCGGCTGCCGTTGCCGGCGGGGGGGCT
 R H S W S R P L C S P L G R R R' A S
 P S V V V A A P V V A L G P A A E P G F
 T L G R G R C A R R C A G A G G G P R L
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 R S S T V R T R T S P A P W C A Q P R
 G R H R R * G P E R H R L R G V H N R G
 V V I D G K D Q N V T G S V V C T T A A
 AGGTCTCATCGACGGTAAGGACCAAGAACGTCACCGGCTCCGTGGTGCACAACCGCGG
 1270 1280 1290 1300 1310 1320
 TCCAGCAGTAGCTGCCATTCCCTGGTCTTGCACTGGCGAGGCACACGTGTTGGCGCC
 P R * R R Y P G S R * R S R P T C L R P
 T T M S P L S W F T V P E T T H V V A A
 D D D V T L V L V D G A G H H A C G R G

FIGURE 6 (CONT'D)

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P	A	M	S	T	S	R	S	A	G	R	R	P	A	L	P	P	C	S	P
R	Q	C	Q	H	R	D	R	R	G	D	R	H	C	R	R	A	H	R	
G	N	V	N	I	A	I	G	G	A	A	T	G	I	A	A	V	L	T	D
CGGGCAATGTCAAACATCGCGATCGGGGGGGGGGGGGCATTCGGGCAATTGGCGCCGGTGCACCG																			
1330	1340	1350	1360	1370	1380														
GGCCGTTACAGTTGCTAGGGCTAGCCCCGGCTGGCCGTAAACGGGGCACGAGTGGC																			
R	C	H	*	C	R	S	R	R	P	P	S	R	C	Q	R	R	A	*	R
P	L	T	L	M	A	I	P	P	A	A	V	P	M	A	A	T	S	V	S
A	I	D	V	D	R	D	A	P	R	R	G	A	N	G	G	H	E	G	V
T	A	T	L	R	R	*	S	P	L	G	S	V	T	S	T	A	S	R	W
R	Q	P	S	G	G	E	V	R	W	A	R	*	R	Q	R	R	H	A	G
G	N	P	P	E	V	K	S	V	G	L	G	N	V	N	G	V	T	L	G
ACGGCAACCCCTCCGGAGGGTAGTCCTGGCTGGCTAACGGTCAACGGCTCACGGCTGG																			
1390	1400	1410	1420	1430	1440														
TGCGGTGGGAGGGCCTCCACTTCAGGCAACCCGAGGCCATTGGCAGTTGGCGGACCC																			
R	C	G	E	P	P	S	T	R	Q	A	R	Y	R	*	R	R	*	A	P
P	L	G	G	S	T	F	D	T	P	S	P	L	T	L	P	T	V	S	P
A	V	R	R	L	H	L	G	N	P	E	T	V	D	V	A	D	R	Q	S

FIGURE 6 (CONT'D)

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D	T	R	R	A	P	D	R	V	T	P	R	Q	P	R	T	A	A	T	T
I	H	V	G	H	R	T	G	*	R	L	G	N	Q	G	R	Q	P	L	Q
Y	T	S	G	T	G	Q	G	N	A	S	A	T	K	D	G	S	H	Y	K
GATACACGTGGCACCCGACAGGGTAACGCCCTCGGCAACCAAGGACGGCAGCCACTACA																			
1450	1460	1470	1480	1490	1500														
CTATGTGCAGCCCCGTGGCCTGTCCCATTGGGAGCCGTTGGCTCCTGCCGGTGTGATGT																			
I	C	T	P	C	R	V	P	Y	R	R	P	L	W	P	R	C	G'	S	C
Y	V	D	P	V	P	C	P	L	A	E	A	V	L	S	P	L	W	*	L
V	R	R	A	G	S	L	T	V	G	R	C	G	L	V	A	A	V	V	L
R	S	L	G	P	L	P	G	S	T	W	P	T	R	C	H	R	*	T	S
D	H	W	D	R	Y	R	G	R	H	G	Q	P	D	V	T	G	E	Q	V
I	T	G	T	A	T	G	V	D	M	A	N	P	M	S	P	V	N	K	S
AGATCACTGGGACCGCTTACCGGGTCGACATGGCCAACCCGATGTACCGGTGAACAAGT																			
1510	1520	1530	1540	1550	1560														
TCTAGTGCACCTGGCGATGGCCCCAGCTGTACAGTGGCACTTGTCA																			
S	*	Q	S	R	*	R	P	R	C	P	W	G	S	T	V	P	S	C	T
I	V	P	V	A	V	P	T	S	M	A	L	G	I	D	G	T	F	L	D
D	S	P	G	S	G	P	D	V	H	G	V	R	H	*	R	H	V	L	R

FIGURE 6 (CONT'D)

R S K S R * P V P N L K R V D A G C E Q
 V R N R G D L F L T * S V S M R A V N S
 F E I E V T C S] * P K A C R C G L * T A
 CGTTCGAAATCGAGGTGACCTCCTAACCTAAAGCGTGTGAACAG
 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
 GCAAGCTTAGCTCCACTGGACAAGGATTGGCATCGCACAGCTACGCCGACACTTGTC
 T R F R P S R N R V * L T D I R A T F L
 N S I S T V Q E * G L A H R H P S H V A 21/43
 E F D L H G T G L R F R T S A P Q S C R
 R V G A G Q S G L A R R F E R L P S V
 A S E P G S Q A * R G D D S S G C H P S
 R R S R A V R P S A A T I R A V A I R Q
 CGCGTCGGAGCCGGCAGTCAGGCCCTAGGCCGACGGATTGAGCGGTGCCATCCGTC
 1630 1640 1650 1660 1670 1680
 GGGCAGGCCTCGGGATCGCCGGCTGCTAAGCTCGCCAACGGTAGGCAG
 A D S G P L * A * R P S S E L P Q W G D
 R R L R A T L G L A A V I R A T A M R *
 T P A P C D P R A R R N S R N G D T L

FIGURE 6 (CONT'D)

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K W Q P H R K L G I S G * A T H' G D R S
 S G N R T A N S V Y P G E L L T V I V P
 V A T A P Q T R Y I R V S Y S R * S F F
 MAGTGGCAACCGCACCGAAACTCGGTATATCCGGTACTCACGGTGAGCTCAGGTGATCGTTCC
 1690 1700 1710 1720 1730 1740
 TTCACCGTTGGCGTTGAGCCATATGGCCCACTCGATGAGTGGCACTAGCAAAGG
 L P L R V A F E T Y G P S S V T I T G
 T A V A G C V R Y I R T L * E R H D N R
 H C G C R L S P I D P H A V * P S R E T
 V V R L D H S G D D R Q A E P G A T G L
 L C A L T T A E T I A R P S P V L P A W
 C A P * P Q R R S P G R A R C Y R L G
 GTTGTGGCTTGACCAACAGCGGAGACGATGCCAGGCCAGGGCTACCGGCTRG
 1750 1760 1770 1780 1790 1800
 CAAACACGGAAACTGGTGTGGCTCTGCTAGCGGTCCGGCTCGGGCACCAGGGCGAAC
 N H A K V V A S V I A L G L G T S G A Q
 Q A G Q G C R L R D G P R A R H * R S P
 T R R S W L P S S R W A S G P A V P K A

FIGURE 6 (CONT'D)

A G P * R I A A G E P L E N L G L Q R G
 R D R D V S P R A N R S K T S D C S A A
 G T V T Y R R G R T A R K P R T A A R P
 GCGGACCGTGTGACGGTATGCCGGCGAACCGCTCGA
 1810 1820 1830 1840 1850 1860
 CGCCCTGGCACTGCATAAGCGGGCGCCGCTTGGAGC
 R S R S T D G R A F R E F V E S Q * L A A
 P V T V Y R R P R V A R F G R V A A R G
 P G H R I A A P S G S S F R P S C R P R G^{23/43}
 R N T R P I V D H I Q H D V R R P G A Q
 G I P G P L S I T C S T T C V G P V L K
 E Y P A H C R S P A A R R A S A R C S S
 CGGAATACCGGCCATTGTCGATCACCTGCAGCACG
 1870 1880 1890 1900 1910 1920
 GCCTATGGCCGGTAACAGCTAAGTGGACGTCGTGCTGC
 P I G P G N D I V Q L V V H T P G T S ' L
 S Y G A W Q R D G A A R R A D A R H E L
 F V R G M T S * R C C S T R R G P A * A

FIGURE 6 (CONT'D)

A R R H D R A G T G V H A G V G Q Q V V G
 R V V T I V P G P V C T R A L A S R L V
 A S S R S C R D R C A R G R W P A G W S
 GCGCGTCGTCAACGATCGTGGCCGGGACCGGGTGTGCACGCCGGCTGGCCAGGCAGGGTTGGT
 1930 1940 1950 1960 1970 1980 24/43
 CGCGCAGGCAGTGCTAGCACGGCCCTGGCCACACGTCGGCCACCGGTCTGGTCCACCA
 R T T V I T G P G T H V R A N A L L' N T
 A D D R D H R S R H A R P R Q G A P Q D
 R R * S R A P V P T C A P T P W C T P *

 H H L V Q P C R I T R D D H R F G G Q V
 T T W C N R A A S P G M T T G S G G R S
 P P G A T V P H H P G * P P V R G A G R
 CACCACCTGGTGCACCGGTGCATCACCCGGATGACCAACCGGTTCTGGGGGGAGGTC
 1990 2000 2010 2020 2030 2040
 GTGGTGGACCGTGGCACGGCGTAGTGGGCCCTACTGGTGGCCAAGCCCCGGTCCAG
 V V Q H L R A A D G P I V V P E P P L D
 G G P A V T G C * G P H G T R P A P R
 W R T C G H R M V R S S W R N P P C T S

FIGURE 6 (CONT'D)

E R P L V I W S G N M S V A D R V N R K
 S A H W * S G P A T * A S L T A S T A S
 A P T G D L V R Q H E R R * P R Q P Q A
 GAGCCGCCACTGGTGATCTGGTCCGGCAACATGAGCGCTGACCGCGTCAACCGCAAG
 2050 2060 2070 2080 2090 2100 2100
 CTCGGGGTGACCACTAGACCAGGGCAGGGACTTGGCAGGGACTTGGGTTC
 L A W Q H D P G A V H A D S V A D V A L
 A G V P S R T R C C S R R Q G R * G C A
 R G S T I Q D P L M L T A S R T L R L G ^{25/43}
 P R H V H R S A F Q R P P R V Q T R Q Q
 R D M S T G P R S S G R P E S R R A S S
 A T C P P V R V P A A A P S P D A P A A
 CCGGCACATGTCCACCGGTCCGGTCCAGGGCCAGTCCAGACGGCCAGGCAG
 2110 2120 2130 2140 2150 2160
 GGCGCTGTACAGGTGGCGGCCAGGTGGCTCGCCGGGGCTCAGGTCTGGCGGTGGTC
 R S M D V P G R E L P R G S D L R A L L
 A V H G G T R T G A A A G L G S A G A A
 R C T W R D A N W R G G R T W V R W C C

FIGURE 6 (CONT'D)

Q Q V L D E T C Y P L G L R C H P A H R
 S R S S T R R V I R S V S D A T R L I A
 A G P R R D V L S A R S P M P P G S S R
 CAGCAGGTCCCTGACGAGACGGTGTATCCGATGCCAACCGGCTCATCGC
 2170 2180 2190 2200 2210 2220
 GTCGTCCAGGAGCTGCTCTGCACAATTAGGCCAGGCCAGGGCTACGGTAGGG
 L L D E V L R T I R E T E S A V R S M A
 A P G R R S T N D A R D G I G G P E D R
 C T R S S V H * G S P R R H W G A * R T^{26/43}
 V C D G L G I V P Y P L R Q F R V T T D
 C A T A S G S S P I R C V N S V * P R I
 V R R P R D R P L S A A S I P C N H G S
 GGTGCGACGGCCTCGGGATCGTCCCCATCCGCTCAATTCCGTGTAAACCACGGAT
 2230 2240 2250 2260 2270 2280
 CACACGCTGCCGGAGCCCTAGCAGGGATAGGGCACGTTAAGGCACATTGGTGCCTA
 H A V A E P D D G I R Q T L E T Y G R I
 T R R G R S R G R D A A D I G H L W P D
 H S P R P I T G * G S R * N R T V V S R

FIGURE 6 (CONT'D)

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R R K G S S Q F M T G I G N E L A H T G	
A A R G V R S S * L A S A T N W R T R V	
P Q G E F A V H D W H R Q R T G A H G F	
CGCCGCAAGGGAGTTGGCAGTTCATGACTGGCATGGCACACGGGT	
2290 2300 2310 2320 2330 2340	
GGGGCGTTCCCCCTCAAGCGTCAAGTACTGACCGTAGCCGTTGCTTGACCGCGTGTGCCCA	
A A L P T R L E H S A D A V F Q R V R T	
G C P S N A T * S Q C R C R V P A G P N	
R L P L E C N M V P M P L S S A C V P K	
F T G L P R R Q C G S D V V E H P V E R	
S L A C R A D S A A A M W S S T R L S A	
H W P A A P T V R Q R C G R A S G * A P	
TTCACTGGCCTGGCGGAGAGTGGCAGATGTCGAGGCATCGGGTGAAGGCC	
2350 2360 2370 2380 2390 2400	
AAGTGAACGGACGGGGCTGTCAACGCCGCTACACGCTAGGCCAACTCGCG	
E S A Q R A S L A A I H D L M R N L A	
* Q G A A G V T R C R H P R A D P Q A G	
V P R G R R C H P L S T T S C G T S R R	

FIGURE 6 (CONT'D)

R P E L P H L G G V C V R F G H P D R
 D P S C P T S V E G F A S G S G T R T G
 T R V A P P R W R G L R Q V R A P G P V
 CGACCCGAGTTGCCCAACCTCGGTGGAGGGTTGGCTCAGGTTGGGCACCCGGACCGG
 2410 2420 2430 2440 2450 2460
 GCTGGGCTCAACGGGGTGGAGCCACCTCCCCAAACGAGTCCAAGCCCCGTGGGCC
 S G L Q G V E T S P N A D P E P V R V P
 V R T A G G R H L P K R * T R A G P' G T
 G S N G W R P P T Q T L N P C G S R Y^{28/43}
 * L D L A A I Q R * V D D F A R G L R D
 S L T S P R S N G R S T T S L A V C A T
 A * P R R D P T V G R R L R S R F A R P
 TAGCTTGACCTCGCCGGATCCAACGGTAGGGTCAACGACTTCGCTCGGGTTGGCGAC
 2470 2480 2490 2500 2510 2520
 ATCGAACTGGAGCGGGCTAGGTTGCCATCCAGCTGCTGAAGCGAGGCCAACGCGCTG
 L K V E G R D L P L D V V E S A T Q A V
 A Q G R R S G V T P R R S R E R N A R G
 S S R A A I W R Y T S S K A R P K R S R

FIGURE 6 (CONT'D)

FIGURE 7

1 GAA TTC CAA CCC TCG GTG CAG ATC CAG GTC TAT CAG GGG GAG CGT GAG 48
Glu Phe Gln Pro Ser Val Gln Ile Gln Val Tyr Gln Gly Glu Arg Glu

49 ATC GCC GCG CAC AAC AAG TTG CTC GGG TCC TTC GAG CTG ACC GGC ATC 96
Ile Ala Ala His Asn Lys Leu Leu Gly Ser Phe Glu Leu Thr Gly Ile

97 CCG CCG CCC CGG GGG ATT CCG CAG ATC GAG GTC ACT TTC GAC ATC 144
Pro Pro Ala Pro Arg Gly Ile Pro Gln Ile Glu Val Thr Phe Asp Ile

145 GAC GCC AAC GGC ATT GTG CAC GTC ACC GCC AAG GAC AAG GGC ACC GGC 192
Asp Ala Asn Gly Ile Val His Val Thr Ala Lys Asp Lys Gly Thr Gly

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FIGURE 7 (CONT'D)

3 AAG GAG AAC ACG ATC CGA ATC CAG GAA GGC TCG GGC CTG TCC AAG GAA 240
Lys Glu Asn Thr Ile Arg Ile Arg Ser Gly Leu Ser Lys Glu 31/43

11 GAC ATT GAC CGC ATG ATC AAG GAC GCC GAA GCG CAC GCC GAG GAG GAT 288
Asp Ile Asp Arg MET Ile Lys Asp Ala Glu Ala His Ala Glu Glu Asp

19 CGC AAG CGT CGC GAG GGC GAT GTT CGT AAT CAA GCC GAG ACA TTG 336
Arg Lys Arg Arg Glu Glu Ala Asp Val Arg Asn Gln Ala Glu Thr Leu

7 GTC TAC CAG ACG GAG AAG TTC GTC AAA GAA CAG CGT GAG GCC GAG GGT 384
Val Tyr Gln Thr Glu Lys Phe Val Lys Glu Gln Arg Glu Ala Glu Gly

FIGURE 7 (CONT'D)

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385	GGT TCG AAG GTA CCT GAA GAC ACG CTC AAC AAG GTT GAT GCC GCG GTG	432
	Gly Ser Lys Val Pro Glu Asp Thr Leu Asn Lys Val Asp Ala Ala Val	
433	GCG GAA GCG GAA GGC GGC ACT TGG CCG ATC GGA TAT TPC GGC CAT CAA	480
	Ala Glu Ala Glu Gly Gly Thr Trp Arg Ile Gly Tyr Phe Gly His Gln	
481	GTC GGC GAT GGA GAA GCT GGG CCA GGA GTC GCA GGC TCT GGG GCA AGC	528
	Val Gly Asp Gly Glu Ala Gly Pro Gly Val Ala Gly Ser Gly Ala Ser	
529	GAT CTA CGA AGC AGC TCA GGC TGC GTC ACA GGC CAC TGG CGC TGC CCA	576
	Asp Leu Arg Ser Ser Ser Gly Cys Val Thr Gly His Trp Arg Cys Pro	
577	CCC CGG CGG CGA GCC CGG CGG TGC CCA CCC CGG CTC GGC	615
	Pro Arg Arg Arg Ala Gly Arg Cys Pro Pro Arg Leu Gly	

FIGURE 8

5' TCGAACGGGGUGTGAACCGGGGCTTCTTGACACTGGCATAGGGGAGTGCTAAG
 3' AGCTTGCTCCCCGCACTGGCCACTGGCAAGAACGTGAGCCGTATCCGCTCACCGATTC
 10 20 30 40 50 60
 AATAACGTTGGCACTCGGACCCGGTAGTGGCTAGGTGGCACGGTGAGGGCAGGGCGTC
 TTATTGCAACCGTGAAGCCACTACGGATCCAGCCCTGCCACTCGCAACTCCGGTCCCCAG
 70 80 90 100 110 120
 GTCCGAGGGAGTGGCAGGGACAACTTGAGCCGTCGGCTCCGTGGGACTTGAGGGGGCCC
 CAGGGCTCACCGCTGGCTGGCAGGGCAGGGCAACTGGGGCAACTGGGGCTAGGGCTCT
 130 140 150 160 170 180
 * R G C R H P V T P V S S P I R R
 AGCGTAAGTAGCCGGGTTGGCTCACCGGGIGACCCCCGGTTCAICCCCCGATCCGGAGGA
 TGGCATTCACTGGCCCCAACGGGAGTGGGGCAACTGGGGAAAGTAGGGGCTAGGGCTCT
 190 200 210 220 230 240
 N H F A M A K T I A Y D E A R R G L E
 ATCACTTGCAATGGCCAAGACAATTGGGTACGGACCAAGAGGGCTGGGCTGGGAGCTCG
 TAGTGAAAGGGTITACGGTCTGTITAAACGCAITGCTGGCTTACCTGCTTACGCTG
 250 260 270 280 290 300
 R G L N A L A D A V K V T L G P K G R H
 GGGGGCTTGAACCGCCCTGGGATGGGTAAGGTGACATTGGGCCCCAAGGGGGCAACCG
 CCCGGAACTTGCGGGAGGGCTACGCCAATTCCACTGCAACGGCTTACGCTGCTTACGCTG
 310 320 330 340 350 360
 V V L E K K W G A P T I T H D G V S I A
 TCGICCTGGAAAAGAAGTGGGTGGGGTGGGATGGGATGGGCTGGGCTGGCTTACGCTGCT
 ACCAGGACCTTCTTCACTGGGGCTGGCTTACGCTGCTTACGCTGCTTACGCTGCTTACGCTG
 370 380 390 400 410 420

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FIGURE 8 (CONT'D)

K E I E L E D P Y E K I G A E L V K E V
 AGGAGATCGAGCTGGAGGATCCGTACGAGAAGATGGGGCCCAGCTGGTCAAAGAGGTAG
 TCCCTAGCTCGAACCTCCATTGGCATGCTCTTAGCCATGCTAGCTTCAGCTGGCTCCATC
 430 440 450 460 470 480

 A K K T D D V A G D G T T A T V L A Q
 CCAAGAACCGATGACCTGGCTGGGGTGAACGGCACCCAGGACGGGACCCGACGGGAGG
 GGTCTTTCTGGCTACTGGCAAGGGGCACTGGCTGGTGGTGGCTGGGGTGGCACGGGTCC
 490 500 510 520 530 540

 A L V R E G L R N V A A G A N P L G L K
 CGTTGGTTCCGGAGGGCCCTGGCAACGTCGGGGGGGGCAACCCCCGGCTGGCTCAAAC
 GCAACCAAGGGCTCCGGACGGGTTGGCAAGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
 550 560 570 580 590 600

 R G I E K A V E K V T E T L L K G A K E
 GGGCATCGAAAGGGGGGGAGGAAGGGTACCCGAGACCCGCTCAAGGGGGCAAGGGAGG
 CCCCTACCTTTCCGGCACCTCTCCAGTGGCTGGCTGGGACGAGTTCCGGGGTTCCTCC
 610 620 630 640 650 660

 V E T K E Q I A A T A I S A G D Q S I
 TCCAGACCAAGGAGGAGATTGGGGCAACGGGAGGGCAATTGGGGGGTCAACCAGCTCCATCC
 AGCTCTGGCTCCGTCTAACGGGGCTGGCTGGCTGGCTGGGAGCTGGTCAAGGTAGC
 670 680 690 700 710 720

 G D L I A E A M D K V G N E G V I T V E
 GTGACCTGATCGCCGAGGGATGGACAAGGGGGCAACGGGGGCGICATCACCGGGGAGG
 CACTGGACATAGGGGCTCCGGCTACCTGTTCCGGAGTGGCAAGTGGCAGCTCC
 730 740 750 760 770 780

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FIGURE 8 (CONT'D)

E S N T F G L Q L E L T E G M R F D K G
 AGTCCAAACACTTTGGCTTGAGCTCGAGTCACCGAGGGTATGGGTATGGCAAGGGCT
 TCAGGGTTGTGGAACCCGACCTGGACTCGAGCTCGAGTCACGCCATACGCCAAGCTGGTCCCAG
 790 800 810 820 830 840

 Y I S G Y F V T D P E R Q E A V L E D P
 ACATCTGGCTACTTCGCGACCCGGAGGGCTCAGGAGGGGCTGGAGGACCCCT
 TGTAGGCCCCATGAAGGACTGGCTGGCTGGAGTCCTGGAGGCCAGGACCTCCGGAGGGAA
 850 860 870 880 890 900

 Y I L L V S S K V S T V K D L L P L L E
 ACATCTGGCTGGCTCACGGTCCACTGCTCAAGGATCTGGCTGGCTGGAGA
 TGTAGGACGACCAGTGGAGGTCCACAGGTGACAGTTCAGGACGGAGGCTT
 910 920 930 940 950 960

 K V I G A G K P L L I I A E D V E G E A
 AGGTCAICGGAGGCCGTAAGCCGGCTGGCTGATCATGCCGAGGACGGTGGGGGG
 TCCAGTAGCCCTGGCCATTGGGACGGACTAGTAGGGCTCTGCAGCTCCGGCG
 970 980 990 1000 1010 1020

 L S T L V V N K I R G T F K S V A V K A
 TGTCACCCCTGGCTGGTCAACAAGAACGGCAACCTTCAAGGTCGGGCAAGGCTC
 ACAGGGTGGGACCAAGCAGTTGGCTAGGGGGCTAGGACGGTCCCTACGGTAAGAGTGGCCACAG
 1030 1040 1050 1060 1070 1080

 P G F G D R R K A M L Q D M A I L T G G
 CGGGCTTCCGGGACCCGGCAAGGGCATGGCTGGAGATAGGCCATTCACGGGGTC
 GGGCGAAGCCCTGGGCTGGGGCTGGCTAGGACGGTCCCTACGGTAAGAGTGGCCACAG
 1090 1100 1110 1120 1130 1140

 Q V I S E E V G L T L E H A D L S L L G
 AGGGTCAAGCGGAAGGAGGCTGGGCTGACGGCTGGAGAACGGGGACCTGCGCTGGCTAGGCA
 TCCACTAGTCGGCTTCTCCAGGGGACTGGGACCTGGGACCTGGACAGGGACGGATCCGT
 1150 1160 1170 1180 1190 1200

FIGURE 8 (CONT'D)

FIGURE 8 (CONT'D)

L E P G V V A E K V R N L P A G H G L N
 TGGAGCCGGGCGTGGGTGGAGAAGGTGGCCAAACCTGGCTGGCCACCGGACTGAACG
 ACCCTGGGCCCCACCCACCCACCCACCTCTTCACCGGTTGGACGGCCGACCGGTGGCTGACTTGC
 1650 1660 1670 1680

A Q T G V Y E D L L A A G V A D P V K V
 CTCAGACCGGGTGTCTACGAGGATCTGGCTCGCTGGCTGGCTGACCCGGTCAAGGTGA
 GAGTCCTGGGCCCCACAGATGCTCTTAGACCGAACGGGAGACGGACTGGCCAGTTCACCT
 1690 1700 1710 1720 1730 1740

T R S A L Q N A A S I A G L F L T T E A
 CCCGGTTGGGGCTGGCAGAAATGGGGCTCCATGGGGCTGTTCCTGACCCACCGAGGCC
 GGGCAAGGGGGGACGGTCTTACGGCCAGGTAGGGTGGCTGGCTGGCTGGCTGGG
 1750 1760 1770 1780 1790 1800

V V A D K P E K E K A S V P G G D M G
 TCCGTTGGGCAAGGGGGAAAGGAGAAGGCTTCCGTTCCGTTGGGGCATGGGTG
 AGCAACGGGCTGGCTGTTCCCTCTCCGAAGGGCAAGGGCACCGGGCTGTACCCAC
 1810 1820 1830 1840 1850 1860

G M D F *
 GCATGGATTCTGACCCCCGGAGAAGTCGGAGGGAGCCGGCTTGTGGGGCC
 CGTACCTAAAGACTGGGGGGCTCTCAGGGTGGCTCTCCAGGGAAACACCCGG
 1870 1880 1890 1900 1910 1920

GGGCTCCCTGGGGAGCTACGGTAACGGAGAAACACCAAGGCAAGTCGGTAGGGCAACCTT
 CCCGAGGAGACCAACCTCGATGCCATGGCTCTTGTGGTGGCTCAGCACATCCGTTGGAA
 1930 1940 1950 1960 1970 1980

TGGCCGGCTGTGGGGAGCTGGGGGGGGCTCGGTGGAGACGGCAAGGGTACGA
 ACCGGGACACCCCCGGCTCAGCCCCGGAGGGCACGGTGGCTACCCATGCT
 1990 2000 2010 2020 2030 2040

FIGURE 8 (CONT'D)

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CACCGGAGGGGGGTGCGTCAATGGGGCTGGGACGGCCTGGGACGGCGT
GTTGGGTGCCCCACAGCACTGGGGACGGAGGGTGGCCAGCT
2050 2060 2070 2080 2090 2100
CGATCAGCGAAGTAGCCGCTAGGAATCGGATGGGGCACAAACAGGGTGACTTCGCTGCCGT
GCTAGTGGCTCATGGCGATCCTAGCCTACCCCCGGTGTGTTGCTCCACTGAAGGGACGCCA
2110 2120 2130 2140 2150 2160
GGGCCAGGTTTGGCGGTACGGACCCCCGATCAGGGCGACGTCGACCACTGGGGGGTC
CCCAGTCCAAAACGGGGCATGGGGCTAGTCCCCGCTGCAAGCTGGTGACCCCCCAG
2170 2180 2190 2200 2210 2220
CATCGGGGGCGTGGGGAGTTGGGACCCGGCTGGACTGCCACCCGTGTGCACCGCGAT
GTAGCCCCGGAGCCCCCTCAAGGGCGTGGGGAGGTGAGCTGAGCTGGCACACGGTGGCTA
2230 2240 2250 2260 2270 2280
2290 2300 2310 2320 2330 2340
GGCCATCATGACGGGTGATCAGGTAAGCGAACGGGTAGICGGCAAGGGGGCCAGGCC
CGGGTAGTAGCTGGCACTAGGCCATTGGCTTGGCCATAGGCCCTATGGGGCTGG
2350 2360 2370 2380 2390 2400
GTTTGAGGTCTACCTTTGGCACCCACGGATTGGGATAAGGGGATGTGTGTTCATC
CAAACCTCCAGATGGAAAAACCGTGGTGGCTAAGCTCTATGGGGCTACACAATGAG
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
CGAACCGGACGGGCTGGGGATTCGGGCTGGGCGTGGGATGGCTGGGCTGGGCTGGG
GCTTGGCTGGGGAGGGCTAGGGGGGACGGCATCCGCTTAAGGCCAGGGGGAGCC
S G V P Q G I R P S A Y A S E R D P S P
* E
Y F H S K P I G S G P 1 S P E R V A T H

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FIGURE 8 (CONT'D)

FIGURE 8 (CONT'D)

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2960 2980 2970 2980 2990 3000
 GGTCCCCGATGCCGCTGTTCAAGGGAGCCGAATTCCCCGATGCCGATGTTTCCCCTGCCGAA
 CCAGGGCTACGGCGACAAAGTCCTCGGCTTAAGGGCTACGGCTACAAAGGGCAGGGCT
 T G I C S H L S G S H G I G I H G S G S

 3010 3020 3030 3040 3050 3060
 GTTGAATAAGCCGACCGTGGCGGTGCCGAGTTCCCGAAGCCGATGTTGCCGCTACCCGA
 CAACTTATCCGGCTGGCAACGGCCACGGCTCAAGGGCTTGGCTACAAACGGGATGGCT
 H F L G V H G T G S H G I F G I N G S G S

 3070 3080 3090 3100 3110 3120
 GTTGAAGCCGGAAACCCATCTGGTATCACCGGTGATCCCGAACCCGATATCCCGAT
 CAACTTGGGGCTTGGTAGACCACACTAGTGGCCACTAGGGCTTGGCTATAAGGGCGA
 H F G G F M Q H D G T 1 G F G I H G S

 3130 3140 3150 3160 3170 3180
 ACCGGGTGTGCCGAAGCCGATATTCCCGTGGCGAGGGTGGCTGGCCAGGGCCAGGG
 TGGCCACAAACGGCTTGGCTATAAGGGCAGGGCTAACGGCTCCAAACGGGA
 G T H G F G I H G D G L H G L N G L H H

 3190 3200 3210 3220 3230 3240
 GCGGGGTGTGCCGCTGCCGATGTTGCCGCTGCCGCTGTTGCCGATGCCGATGCCGAA
 CGCCCAACAACGGGCACGGCTACAAACGGCCACGGCCACGGCTAACAAACAA
 G T H G S G I H G T G S H G S H H H

 3260 3260 3270 3280 3290 3300
 GCCGATGTTGGTGGCCGATGTTGTTGCCGATGTTGCCGCTGCCGCTGCCGAA
 CGGCTACAAACAACGGCTACAAACAAACGGCTAACAAACGGCTAACAAACGGCT
 G I H N N G I H H H G I H G S G T N G F

 3310 3320 3330 3340 3350 3360
 GCCCAGATGATCTGGCGGTCTTGGCATGTCGATGCCGAGGGTTCCGCAAGACCTGCTG
 CGGGTCTAACTAGACGGCGAACAAACGGCTACAGCTAACGGCTAACAGCTAACGG
 C I H I N G I H R I V H R I V Q H N F

FIGURE 8 (CONT'D)

FIGURE 8 (CONT'D)

3790 3800 3810 3820 3830 3840
 GGGCGGCAATTAGCCGGGCCGGACCCGGAAACATCAGTCGGGAATTGAACTC
 CCGGGCTCGTTAATGCCAGGGCTGGCCCTGGGGTTAGTCACGGCTTAACTAGAG
 A A I L P G S P A F M L A S H I E
 3850 3860 3870 3880 3890 3900
 TGGCGGCAACCACGGAAAATGCCGGCTTGTCAAGGGATCCAACCTAACCTGTCAGCGACCC
 ACCGGCGCTTGTGGCTTTACGGGGAAACAGTCGGCTTAGGTTGAATTGAGCTGGCTGGC
 P P L W A F H P S T L R D L K V T L S R
 3910 3920 3930 3940 3950 3960
 TTGGCGGGTATCGGCACCTCAATTACCACTCATCTTGGGGTCACTTTGGAGGCC
 AACGGCACCGCCATAGCCGTCAAGTTATGGTGAGTAGAAACCCAGTAGAAACCTCGGG
 Q R P I P V E I G S H K P T M K P A G
 3970 3980 3990 4000 4010 4020
 CCTAGGAACCCAGCTTACCTTAGTCGGGTAGGGGGACTTGGGGGGATGCAAGC
 GGATCCTTGGGGTCAAATGGATCAGGGCCATCCCCGGCTGACGGGGCCCTACGTCG
 R P V A L K G L G P Y P G V P R S A A
 4030 4040 4050 4060 4070 4080
 TGAGGGCTGCCCGCTGTAATGTCGGCTGGTAAGGCAAGGCAAGGGCGGGCGCC
 ACTCCAGACGGTGGACGGCATTACGGGACCATACCCGCTGGCTGGGCGCC
 S P R G C A G Y H R Q P L C R R P G
 4090 4100 4110 4120 4130 4140
 AAGAGTTGGCTCCGGGACGGGTTCACCCGGTTGATCGAACATGTCGACGAACCTCACCGGAGC
 TTCTAACGGGGCTGGCAAGTGGCCAAACTAGCTTGTACAGCTGTTGAGTGGCTGC
 L T A G R R T *
 4150 4160 4170 4180 4190 4200
 GCCTCACCGACCAACTCGCTGGCTGGGACCCCCGACGGCAACAGGATTTGGGIGGG
 CGGAGTGGCTGGGTGAGGGACGAATGGGGCTGGGGTGGGCTGGGTTGTCGTAACGGACCC

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FIGURE 8 (CONT'D)

4210 4220 4230 4240 4250 4260
TGCTCTGGCACAGCGCCGGGTGCAGGATAACAGGICGCCATGTGCCGGCGTGGAAAG
ACGAGAACCGGTGTGGGGCCACGGTCTATACTGTCCAGGGGTACACGGGG
4270 4280 4290 4300 4310 4320
AGGTGTGGACCCGGGACGGGTTGGGTGGACCCGGCTTGGGTTAGATCTGCCGGCAGGACA
TCCACACCTGGGGGCTGGCAACCCACCTGGGAAACCCAAATCTAGACGGGG
4330 4340 4350 4360 4370
CCGGATAATGGACACCCGTCCCCGAGGATGTGGCGAAGGTACGGGCACCGGAATTCT
GGCCTATACCTGTGGCAGGGCTCCATGCCCTACACCGCTTCATGCCCGTGGGGCTGGCTTAAG
3'
5'